**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re application of:

WARREN WARD

Group Art Unit: 1595

Examiner: Tigabu Kassa

Serial No.: 10/595,033

Filed: January 4, 2006

For: COMPOSITIONS COMPRISING COMPONENTS COATED WITH A LIQUID IMPERMEABLE BUT GAS PERMEABLE LAYER, USE THEREOF FOR TREATING CUTANEOUS AND OTHER EXOCRINE GLAND DISEASES

Attorney Docket No.: WAW0101PUSA

# DECLARATION OF WARREN WARD
# UNDER 37 C.F.R. § 1.132

Commissioner for Patents
U.S. Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450

I, Warren Ward, do hereby declare and state as follows:

1. I graduated from UK Grammar School with certificates in English, French, English Literature, Pure Maths, Applied Maths, Physics, Chemistry, Art, and Advanced Level Pure Maths and Applied Maths.

2. My professional background is one of executive or general manager, with employment associated with the management of large holiday centres in the UK from 1964 to 1979. From 1979 to 1996, I held a Chief Officer appointment in UK Local Government. From 1996 to 2000, I lectured in information technology at Llandrillo College, UK. I was also a Fellow of the Institute of Trade Valuers UK, and pursued that profession until 2002. From 1984 onwards, I undertook part time investigation into the health status of UK citizens and the etiology of chronic conditions. By 2002 this became a full time occupation.

3. I am the sole inventor of the claimed subject matter of U.S. Application Serial No. 10/595,033, hereinafter the "Application" and am familiar with the content of the Application and the Office Action dated October 5, 2010 received from the United States Patent and Trademark Office, hereinafter the "Office Action."

4. I understand the pending claims 7-11, 24-28, 30-33 and 36 are rejected under 35 U.S.C. § 101 as lacking patentable utility (Office Action, pages 2-3). In particular, the Examiner opines that "there is insufficient evidence to show that a compound which is not released on or into the body can have any medically beneficial effect." *Id.*

5. The pending claims in current form are directed to a preparation for use as a medicament. The claimed preparation is not a conventional pharmaceutical composition or compound that must generally be dissolved into body circulation for cellular uptake and subsequent metabolization. See for instance Applicants' Amendment dated September 23, 2009 at pages 7-10.

6. The claimed preparation is at least partly based on the understanding that all living cells sense and respond to their environment by means of mechanisms known as cell signaling pathways. These mechanisms are part of a complex system of communications that govern basic cellular activities and coordinate the actions of cells.

7. Cell signaling refers to the process by which extracellular substances produce an intracellular response. This is an essential and widespread biological phenomenon by which hormones, neurotransmitters, and other agents regulate cellular function. In some cases, the natural agent inducing the response is present on the surface of a nearby cell, or is present in the extracellular matrix on which cells reside.

8. From 2001 onwards, I set out to experiment and investigate the creation of an exogenous signaling pathway as a therapeutic method of cell signaling via epithelial surfaces and thus activating total body cellular signaling. This therapeutic method is particularly useful for chronic conditions where the physiology has moved away from

normal natural interactive controls or homeostasis, e.g. essential hypertension. There are five great advantages of this method of treatment:

- Molecules can be used to affect cell signaling without the molecules having to be dissolved in body fluids.
- Molecules can be used to reinstate normal homeostasis of humans and animals with chronic conditions, in contrast to conventional drugs which often interfere with normal physiological processes.
- Molecules which are found naturally in the body can be used to beneficially affect cell signaling pathways.
- Since, in contrast to conventional drugs, there is no dissolution or elimination, the molecules can be carefully targeted to avoid all side effects, creating a high level of safety.
- Molecules, including established therapeutic molecules, can be used to affect cell signaling pathways more effectively, but much more safely than conventional drugs.

9. I reasoned that the creation of an exogenous signaling pathway would require both the presence of introduced molecules in proximity to the epithelial surfaces, and transient presentation of such chemicals in microscopic quantities, or apparent microscopic quantities, to the cells of the epithelial surfaces. Induced changes in their immediate environment could be calculated to elicit a response by the epithelial cells.

10. After a large number of experiments, it was found that a rapid response could be achieved by transient presentation of appropriate chemicals to the epithelial surface. A working method was determined to enclose chemicals within a microscopically perforated robust coating together with arrangements to ensure that these coated chemicals had constant movement, or apparent constant movement. A number of coated products can be formulated in this way. I termed this methodology 'Smart Cell Signal'™ .

11. My research and elucidation of the 'Smart Cell Signal'™ led to the development and manufacture of the unique Equiwinner™ patches.

12. As recited in the pending claims with particular reference to the new claim 37, the said Equiwinner™ patches are constructed to have two spheres containing sodium chloride mounted on one side of the patch and spaced apart, and the sodium chloride

is coated with an aqueous liquid impermeable but gas permeable layer comprising white beeswax hardened with talc and cornstarch, all as set out in the Application.

13. Equiwinner™ patches were originally designed to activate angiogenesis of skin capillaries so as to reduce the excessively high blood pressure of racehorses under maximum exertion. This high pressure was known to cause exercise induced pulmonary haemorrhage (EIPH), or horse bleeding. This is a very common problem in horses. Equiwinner™ also corrects anhidrosis, or non-sweating, another previously untreatable condition in horses, particularly those in hot climates. The 'Smart Cell Signal'™ is also effective throughout the body in restoring full hydration of horse body tissues, indicating persistence of the induced cell to cell signaling. Another condition for which Equiwinner™ is rapidly effective is equine rhabdomyolysis, or azoturia, commonly known as "tying-up".

14. I, the discoverer of the 'Smart Cell Signal'™, believe that I have described for the first time in history the full aetiology of the previously unknown cause of automatic head shaking in horses, which can be corrected using Equiwinner™.

15. Further information related to the Equiwinner™ patches may be found at www.equiwinner.com, www.signal-health.com, and www.equinereset.co.nz.

16. The "Equiwinner" patches have been sold to professional horse trainers in various countries including the United States, United Kingdom, Australia and New Zealand. The retail selling price has been between 123 and 130 US dollars (or the equivalent) plus carriage and tax where applicable, for a box of ten patches, plus two spare patches.

17. The Equiwinner™ patches according to the claimed invention and in particular new claim 37, have been very well received by the horse trainers, representing the only effective treatment known for the market. The total number of Equiwinner™ patches sold in the last five consecutive calendar years were 5000 patches, 11.000 patches, 18,000 patches, 24,000 patches and 25,000 patches, with a capital value at year 2010 of several

hundred thousand dollars. The magnitude of the increase in sales is further reflective of the high quality of the Equiwinner™ patches, all constructed according to the claimed invention.

18. In 2005, I supplied a quantity of Equiwinner™ patches, constructed according to the claimed invention and in particular according to new claim 37, to a UK veterinarian Dr. Steve Gittins. Dr Gittins is an equine specialist, a Bachelor of Veterinary Science and a Member of the Royal College of Veterinary Surgeons. In his statement dated 14 July 2005, submitted herewith as Exhibit 1, Dr. Gittins reported a positive medical effect of the said supplied Equiwinner™ patches.

19. Later in 2005, I supplied a quantity of Equiwinner™ patches, constructed according to the claimed invention and in particular according to new claim 37, to an Italian veterinarian Dr. Paola Gulden. Dr Gulden was Vice-President of the learned Societa Italiana Veterinari per Equini (Italian Society for Equine Veterinarians) in year 2005-2007 and President in year 2007-2009. Dr Gulden treated a number of horses having Exercise Induced Pulmonary Haemorrhage by placing on the horses the said supplied Equiwinner™ patches for between three and ten days. In her statement dated December 2005, submitted herewith as Exhibit 2, Dr. Gulden reported that "All of the horse(s) had a clear improvement of the pathological condition."

20. I understand the pending claims 7-11, 24-28, 30-33 and 36 are rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the enablement requirement (Office Action, pages 5-8). In particular, the Examiner opines that substances enclosed within a layer which is not permeable to liquid but permeable to gas cannot have any effect outside the layer when in water. For at least the following, I contend that the said substances can be detected from outside the layer and therefore can influence the environment outside the layer when in water.

21. I made two solid spheres of about 9 mm diameter comprising sodium chloride crystals coated with white wax USP, a pharmaceutical grade of beeswax, hardened with talc and cornstarch as described in the description of the patent application para 0093.

These were each placed on a piece of aluminium, 30mm by 35mm x 2mm labelled A1 and A2, in two glass vessels and immersed in purified water BP. The vessels were covered and left in a room at 22 degrees C for 96 hours. The two spheres remained resting in the same place on the aluminium at this time. They did not float. The same two solid spheres were then carefully examined using both visual and microscopic assessment and found to be unchanged by the immersion in water. The place on the aluminium where the spheres had each been resting was the photographed at about 60 x magnification with the results shown in Exhibit 3A1 and 3A2.

22. On examination and by photography the aluminium was found to be substantially corroded, including the removal of aluminium to a depth of 1mm, only in the place where each of the spheres had been placed. Throughout this document, I intend the word "corroded" or the like to mean that the metal or the metals have been eaten away.

23. I then placed the two spheres on two fresh pieces of the same aluminium of the same size labelled B1 and B2, placed in the two glass vessels and immersed in fresh purified water BP. The vessels were then covered and left in a room at 22 degrees C for 96 hours. The two spheres remained resting in the same place on the aluminium at this time. The two spheres were then carefully examined using both visual and microscopic assessment and found to be unchanged by the immersion in water. The spheres did not float. The place on the aluminium where the spheres had each been resting was the photographed at about 60 x magnification with the results shown in Exhibit 3B1 and 3B2.

24. On examination and by photography the aluminium was found to be substantially corroded, including the removal of aluminium to a depth of 1mm, only in the place where each of the spheres had been placed.

25. I then made two solid cylinders of about 8mm length and 4mm diameter comprising sodium chloride crystals coated with white wax USP, a pharmaceutical grade of beeswax, hardened with talc and cornstarch as described in the description of the patent application para 0093. The two cylinders were each placed on two fresh pieces of the

same aluminium of the same size labelled C1 and C2, placed in the two glass vessels and immersed in fresh purified water BP. The vessels were then covered and left in a room at 22 degrees C for 96 hours. The two spheres remained resting in the same place on the aluminium at this time. The two cylinders were then carefully examined using both visual and microscopic assessment and found to be unchanged by immersion in water. The cylinders did not float. The place on the aluminium where the cylinders had each been resting was the photographed, with each of C1 and C2 requiring two photographs to cover the length of the cylinder, at about 60 x magnification with the results shown in Exhibit 3C1 and 3C2.

26. On examination and by photography the aluminium was found to be substantially corroded, including the removal of aluminium to a depth of up to 1mm, only in the place where each of the cylinders had been placed.

27. I made two spheres of about 9mm diameter comprising white wax USP, hardened with cornstarch and talc. However these were found to float in water and were discarded.

28. I then made two spheres of about 9mm diameter comprising a core of a PVC sphere of about 2mm diameter coated with white wax USP, hardened with cornstarch and talc, as described in the description of the patent application para 0093. PVC was chosen as an inert substance of high enough specific gravity to prevent the spheres from floating. The two spheres were then placed on two fresh pieces of the same aluminium of the same size labelled D1 and D2 placed in the two glass vessels, and immersed in purified water BP. The two vessels were then covered and left in a room at 22 degrees C for 96 hours. The two spheres remained resting in the same place on the aluminium at this time. The two pieces of aluminium were carefully examined using both visual and microscopic assessment and found to be unchanged. I then took the two pieces of the aluminium labelled D1 and D2 and placed these in the two glass vessels. I then added to each vessel 25 ml of purified water BP in which two tenths of a gram of sodium chloride crystals had been dissolved. The vessels were then covered and left in a room at 22 degrees C for 96 hours. The two pieces of aluminium

were then carefully examined using both visual and microscopic assessment and found to be unchanged by being used. The place on the aluminium where the spheres had previously each been resting was the photographed at about 60 x magnification with the results shown in Exhibit 3D1 and 3D2.

29. On examination and by photography the no corrosion of the aluminium was found.

30. The same two spheres comprising sodium chloride coated with white wax BSP hardened with cornstarch and talc, used earlier with aluminium were now placed each on a zinc coated mild steel washer labelled E1 and E2 of about 30mm diameter placed in the two glass vessels and covered with purified water BP. The vessels were then covered and left in a room at 22 degrees C for 24 hours to 48 hours. The two spheres remained resting in the same place on the washer at this time. The two spheres were then carefully examined using both visual and microscopic assessment and found to be unchanged. The place on the washers where the spheres had each been resting was the photographed, with two photographs required to cover the area for E1, at about 60 x magnification with the results shown in Exhibits 3E1 and 3E2.

31. On examination and by photography, the area under the spheres was seen to have had the zinc coating removed. The same two spheres of about 9mm diameter comprising a core of a PVC sphere of about 2mm diameter coated with white wax USP, hardened with cornstarch and talc were placed each one on a zinc coated washer of the same type, labelled F1 and F2 and placed in the two glass vessels immersed in purified water BP. The vessels were then covered and left in a room at 22 degrees C for 24 hours to 48 hours. The two spheres remained resting in the same place on the washers at this time.

32. The two washers were then examined and found to be unchanged by being used. The place on the washers where the spheres had each been resting was the photographed at about 60 x magnification with the results shown in Exhibits 3F1 and 3F2.

33. I have now provided an affidavit as stated above demonstrating that the substance, sodium chloride, enclosed within the liquid impermeable but gas permeable layer, does affect its immediate surrounding environment without the preparation of the invention being changed in any way. The effect on the surrounding metals is solely the result of the construction of the spheres is demonstrated by the observation that a similar amount of sodium chloride to that included in the spheres and cylinders, when dissolved in water has no corrosive effect on the metals. It should be noted that the same two spheres containing coated sodium chloride were used for periods of 96 hours then 96 hours then 48 hours in water and on inspection were not found to be changed in any way. As shown by the above test at para 27, if in fact after the whole of the time in water the sodium chloride had leaked into solution the remaining hardened beeswax layer would have become buoyant and floated upwards. This did not occur. Secondly as previously noted within Exhibit 3 submitted in response to the Office Action of January 7, 2009, "polymer membranes may be highly swollen by a penetrating liquid." Swelling did not occur with the spheres in the test. The spheres remained unchanged. It should additionally be noted that if the sodium chloride had escaped from the coating this could not be responsible for the action on the metals, only the sodium chloride within the layer had that action. The tests further support my assertion that gaseous water molecules are adjacent to the medically efficacious substance, and form a continuous thread of water between the substance, through the gas permeable liquid impermeable coating, to the surrounding liquid water.

34. I further refer to the textbook titled "Permeation of Gases and Vapours in Polymers" previously submitted as Exhibit 2 dated April 7, 2009. This textbook clearly shows why water vapour is attracted through the polymer layer (including a wax layer) so as to be in contact with the substance within the layer, and in use to provide a thread of water between the substance and body cells.

35. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the Application and any patent issued therein.

Signed: Warren Ward

Warren Ward

Date: 4 APRIL 2010

# Premier Veterinary Centre

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**14th July 2005**

**Dear Warren**

**Just to give you some feedback on the Equiwinner patches.**

**I have used them in a number of cases as recommended and have had very good success. I have used Equiwinner for many bleeders (Exercise Induced Pulmonary Haemorrhage), some of which have been chronic cases. So far they are all doing well with no relapses. In one case a second course was needed.**

**A most pleasing case was a fifteen year old cob mare who had been tying up since last November. She is now back in work, doing well and 'looking fantastic'.**

**I have also seen Equiwinner used in showing horses and again the owners are very pleased with the shine they put on the horses and how well they look.**

**I am currently trialling them in poor performance and under performing horses. Although I have only so far used them on four animals, each one has shown an improvement in performance and racing time.**

**Yours sincerely,**

**Steve Gittins**

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# PRELIMINARY CLINICAL TRIAL ON "EQUIWINNER PATCH"

## Introduction

Exercise-Induced Pulmonary Haemorrage (EIPH) is a widespread condition which affects the most breeds of horses undergoing strenuous athletic events. The aetiology and the pathogenesis are still unclear, several theories have been advanced concerning its developing.
Underventilated lung regions, due to small airways disease, can cause an extreme fluctuations in the alveolar pressure, which can results in parenchymal tearing or alveolar capillary rupture (Robinson et al., 1980). Also, small airways disease, coupled with maximal exercise, induces a localized hypoxia, resulting in bronchial angiogenesis, pulmonary vasoconstriction with a greater proportion of bronchial blood flowing to the alveolar capillary bed trough bronchial anastomotic connection. The advancing margin of the new vascular growth of the bronchial vessels, could be indicate as the site of haemorrage. (Clarke, 1985)

Its prevalence is estimate to be between 75% and 26% in the Thoroughbred and in the Standarbred. The prevalence of EIPH increases with the age of the horse, there is no clear correlation between EIPH and the location of stables, condition or type of the track (Pascoe et al., 1981, Voynick et al., 1986). It has been statistically demonstrated that EIPH is a cause of reduced performance in race horses (Hinchcliff, 2004).

Diagnosis is based on the assessment of the presence of blood in the upper respiratory tract. The presence of epistaxis or traces of blood at the nostrils, is usually only present in the most severe cases of EIPH, therefore tracheobronchoscopic examination is considered the elective diagnostic tool (Mac Namara et al, 1990, Birks et al, 2002). Cytologic count on fluid obtained by bronchoalveolar lavage is another method which can give a quantification of the haemorrage (Meyer et al, 1998). The collection of lavage fluid may detect haemorrage that is not evident on endoscopic examination, but this technique is considered to be impractical, being invasive and requiring sedation of the horse and often, administration of local anesthetic solution into the airways (Hinchcliff et al, 2004).

There are no effective treatments for EIPH. A better management of the environment has been recommended, with the aim to reduce any source of dust or mold. Medical treatments, such as corticosteroids to reduce the inflammatory reaction in the lung, bronchodilators to improve ventilation and airway secretions and to increase mucociliary clearence, diuretics (furosemide) to reduce pulmonary

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pressure and resistance, have been and are currently used to try to minimize the negative effects of EIPH associated with poor performances (Ainsworth et al., 2000).

Equiwinner Patch is a device which works on the equine organism to restore normal homeostasis, balancing intra and extra cellular fluids, using a patented messenger cell signalling technology. The device is totally drug free, therefore there is no chemical action on the body of the horse.

In this clinical study Equiwinner Patches have been used, following the instruction of the manufacturer, to treat EIPH in Standardbred horses. This study, which has not the features of a clinical experiment on a scientist basis, aims to give a preliminary idea of the effectiveness of the Equiwinner Patches.

Materials and methods

Five Standarbred horses with a known history of EIPH of different grade of severity, were considered. The horses, three stallions, one female and one gelding, of age between three and six year old, were regularly trained all in the same stable, in the North-West of Italy. All the horses were fed, managed and trained in a similar way.
All the horses were vaccinated and wormed on a regular basis. All the horses were not on any kind of medical treatments during the period of this study.
This study was performed over a period of time of five months, from July to November 2005.

Assessment of severity of EIPH was performed by endoscopic examination of the upper airways, and classification of gravity of EIPH was based on a grading system which consists of five levels (0-4), based upon the amount and location of blood in the upper respiratory tract (Hinchcliff et al., 2004). In one case, (horse C) EIPH was revealed by the bronchoalveolar lavage (BAL) during a previous complete examination on high speed treadmill. The cytological examination of the BAL revealed a number of red cells and of hemosiderophages consistent with recent episodes of haemorrage, despite of the endoscopic examination which revealed no presence of blood (grade 0).

The five horses were divided into two groups. The first group was composed by three horses, two (horses A, B) with history of EIPH of grade 4, and one with history of EIPH of grade 0, but episodes of significant EIPH were revealed by the BAL cytological examination (horse C). The second group (horses D,E) included horses with EIPH from grade 1 to 2.

In the first group, horses were rested for a period of ten days. During this period of time the Equiwinner Patch was applied daily, at the top of the hindquarter, as recommended. Horses were walked by hand or in the walker twice a day. No access to paddocks was allowed to the horses because of the hot climate at the time of the experiment. Horses were gradually put back on normal training at the end of the treatment and raced as soon as they were considered ready for it.

Horses belonging to the second group were kept on normal training and treated with Equiwinner Patches, once a day, for five days prior the day of the race. In one case the course of treatment was reduced to three days, because the declaration of participants was to close to the date of the race. Equiwinner Patch was removed only during the actual exercise, and replaced after the horses were showered and dried.

In both groups, endoscopic examination was performed 45 to 60 minutes after racing, the horses were unsedated and restrained by use of a nose twitch.
As a part of normal health checking during training, all horses were tested for lactate serum level after each session of training.

Results

First group

Horse A: 6yo, bay stallion. Known history of EIPH grade 4.
Very good performance at the first race, ten days after the end of treatment (second after a brake at the start). Endoscopic examination after race revealed no blood in the upper airways (grade 0). Lactate serum level were improved during normal training. At the second race after treatment the horse performance was lower, endoscopic examination revealed presence of mucus and some flecks of blood in trachea (grade 1).

Horse B: 4yo, bay stallion. Known history of EIPH grade 4.
This horse was put back in training and checked at the stable after the first fast training. This horse was used to be trained at this speed after furosemide (Lasix) administration. In this case, with no pharmacological treatment, the endoscopic examination 45 minutes after exercise was completely negative. Also the serum lactate level was under usual values. Good performance on first and second race after treatment, despite the presence of a narrow stream of blood in the trachea at endoscopic examination (grade 1).

Horse C: 6yo, bay gelding. History of poor performances in the last few months, complete clinical examination revealed signs of recent episodes of EIPH at the

BAL cytological count. Never had evidence of blood at the endoscopic examination after exercise.
Very good results at the first and second race after treatment, improved serum lactate level. Endoscopy always negative, unfortunately it was not possible to perform a BAL to check the cytological count. This horse was also definitely in a better general conditions after the period of treatment.

Second group

Horse D: 3yo, chestnut female. History of EIPH grade 2.
Treated twice with a course of five days prior of the day of race. Moderate improvement of performances at both races, presence of flecks and very narrow stream of blood (grade 1), lactate serum level not significantly changed during normal training.

Horse E: 5yo, bay stallion. History of EIPH grade 1 to 2.
Treated twice with a five days course and one with a three days course because declaration of participants was closer to the day of race.
Good performances at the first two races, not very well at the third. Endoscopic examination negative at the first and second time, presence of one long stream of blood in the trachea at the third examination after racing. Presence of flakes of witish mucus suggested the presence of a subclinic respiratory disease.

Finally, horse A, belonging to group one, received other two course of five days the first time prior to the day of race, with the same protocol followed for the group two, about two months after the end of the primary treatment. The horse performed well again, endoscopic examination was of grade 1 on both occasion, with very narrow stream of blood in the trachea (grade 1).

Discussion

Even if the number of horses examined is too low to assess effectiveness of Equiwinner Patches, results are definitely very encouraging. All the horse had a clear improvement of the pathologic condition, particularly the ones belonging to the first group, who received a longer treatment coupled with a period of rest. The association of other stressful factors with poor performances, such as travelling, and the individual capability to react to these factors, needs to be further investigated, to clear up if the uncomplete response to the treatment with Equiwinner patch can be related to these factors. Furthermore, period of rest plays a determinant rule in reducing the severity of EIPH.
Good results were also obtained treating a horse for ten days and giving a shorter course of treatment two or three months later. Further studies are required to optimise the use of this device in treatment and prevention of EIPH.

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Paola Gulden, DMV
Italy, December 2005

pancreatic $\beta$ cells. The drug is absorbed rapidly from the gastrointestinal tract; peak blood levels are obtained within one hour. The half-life of the drug is about one hour. These features of the drug allow for multiple preprandial use, as compared to the classical once- or twice-daily dosing of sulfonylureas. Repaglinide is metabolized primarily by the liver. Metabolites of the drug do not have a hypoglycemic action. Repaglinide should be used cautiously in patients with hepatic insufficiency. Because a small proportion (about 10%) of repaglinide is metabolized by the kidney, increased dosing of the drug in patients with renal insufficiency also should be performed cautiously. As with sulfonylureas, the major side effect of repaglinide is hypoglycemia.

## Nateglinide

*Nateglinide* (STARLIX) is an orally effective insulin secretagogue derived from D-phenylalanine. Like sulfonylureas and repaglinide, nateglinide stimulates insulin secretion by blocking ATP-sensitive potassium channels in pancreatic $\beta$ cells. Nateglinide promotes a more rapid but less sustained secretion of insulin than do other available oral antidiabetic agents (Kalbag *et al.*, 2001). The drug's major therapeutic effect is reducing postprandial glycemic elevations in type 2 diabetic patients. Nateglinide recently has been approved by the United States Food and Drug Administration (FDA) for use in type 2 DM and is most effective if administered 1 to 10 minutes before a meal in a dose of 120 mg. Nateglinide is metabolized primarily by the liver and thus should be used cautiously in patients with hepatic insufficiency. About 16% of an administered dose is excreted by the kidney as unchanged drug. Dosage adjustment is unnecessary in renal failure. Early studies have suggested that nateglinide therapy may produce fewer episodes of hypoglycemia than do other currently available oral insulin secretagogues (Horton *et al.*, 2001).

## Biguanides

*Metformin* (GLUCOPHAGE) and phenformin were introduced in 1957 and buformin was introduced in 1958. The latter was of limited use, but metformin and phenformin were widely used. Phenformin was withdrawn in many countries during the 1970s because of an association with lactic acidosis. Metformin has been associated only rarely with that complication and has been widely used in Europe and Canada; it became available in the United States in 1995. Metformin given alone or in combination with a sulfonylurea improves glycemic control and lipid concentrations in patients who respond poorly to diet or to a sulfonylurea alone (DeFronzo *et al.*, 1995).

Metformin is absorbed mainly from the small intestine. The drug is stable, does not bind to plasma proteins, and is excreted unchanged in the urine. It has a half-life of about 2 hours. The maximum recommended daily dose of metformin in the United States is 2.5 g, taken in three doses with meals.

Metformin is antihyperglycemic, not hypoglycemic (*see* Bailey, 1992). It does not cause insulin release from the pancreas and does not cause hypoglycemia, even in large doses. Metformin has no significant effects on the secretion of glucagon, cortisol, growth hormone, or somatostatin. Metformin reduces glucose levels primarily by decreasing hepatic glucose production and by increasing insulin action in muscle and fat. The mechanism by which metformin reduces hepatic glucose production is controversial, but the preponderance of data indicates an effect on reducing gluconeogenesis (Stumvoll *et al.*, 1995). Metformin also may decrease plasma glucose by reducing the absorption of glucose from the intestine, but this action has not been shown to have clinical relevance.

Patients with renal impairment should not receive metformin. Hepatic disease, a past history of lactic acidosis (of any cause), cardiac failure requiring pharmacological therapy, or chronic hypoxic lung disease also are contraindications to the use of the drug. The drug also should be withheld for 48 hours after administration of intravenous contrast media. The drug should not be readministered until renal function is normal. These conditions all predispose to increased lactate production and hence to the fatal complications of lactic acidosis. The reported incidence of lactic acidosis during metformin treatment is lower than 0.1 case per 1000 patient years, and the mortality risk is even lower.

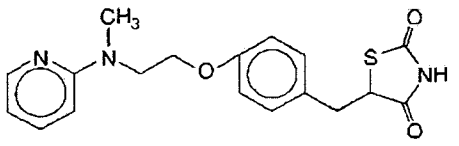
Acute side effects of metformin, which occur in up to 20% of patients, include diarrhea, abdominal discomfort, nausea, metallic taste, and anorexia. These usually are minimized by increasing the dosage of the drug slowly and taking it with meals. Intestinal absorption of vitamin $B_{12}$ and folate often is decreased during chronic metformin therapy. Calcium supplements reverse the effect of metformin on vitamin $B_{12}$ absorption (Bauman *et al.*, 2000).

Consideration should be given to stopping treatment with metformin if the plasma lactate level exceeds 3 mM. Similarly, decreased renal or hepatic function also may be a strong indication for withholding treatment. It also would be prudent to stop metformin if a patient is undergoing a prolonged fast or is treated with a very-low-calorie diet. Myocardial infarction or septicemia mandate stopping the drug immediately. Metformin usually is

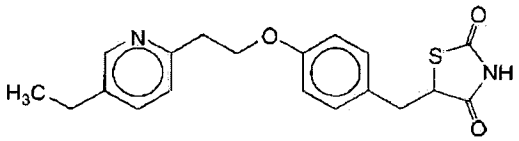
administered in divided doses either two or three times daily. The maximum effective dose is 2.5 g daily. Metformin lowers hemoglobin $A_{1c}$ values to a similar extent as do sulfonylureas (about 2.0%). Metformin does not promote weight gain and can reduce plasma triglycerides by 15% to 20%. There is a strong consensus that reduction in hemoglobin $A_{1c}$ by any therapy (insulin or oral agents) can lead to diminished microvascular complications. Metformin, however, is the only therapeutic agent that has been demonstrated to reduce macrovascular events in type 2 DM (UK Prospective Diabetes Study Group, 1998b). Metformin can be administered in combination with sulfonylureas, thiazolizinediones, and/or insulin. A fixed-combination tablet containing glyburide (glibenclamide) and metformin (GLUCOVANCE) is available.

## Thiazolidinediones

Three thiazolidinediones have been used in clinical practice (*troglitazone*, *rosiglitazone*, and *pioglitazone*). However, the first of these agents to be introduced (troglitazone) has been withdrawn from use because it was associated with severe hepatic toxicity. The structures of rosiglitazone and pioglitazone are shown below.




ROSIGLITAZONE




PIOGLITAZONE

Thiazolidinediones are selective agonists for nuclear peroxisome proliferator–activated receptor-gamma ($PPAR_\gamma$). These drugs bind to $PPAR_\gamma$, which, in turn, activates insulin-responsive genes that regulate carbohydrate and lipid metabolism. Thiazolidinediones require insulin to be present for their action. Thiazolidinediones exert their principal effects by lowering insulin resistance in peripheral tissue, but an effect to lower glucose production by the liver also has been reported. Thiazolidinediones increase glucose transport into muscle and adipose tissue by enhancing the synthesis and translocation of specific forms of the glucose transporter proteins. The thiazolidinediones also can activate genes that regulate free fatty-acid metabolism in peripheral tissue. Studies are in progress to determine if these agents reduce insulin resistance primarily by their actions on free fatty-acid metabolism.

Rosiglitazone (AVANDIA) and pioglitazone (ACTOS) are taken once a day. Both agents are absorbed within about 2 hours, but the maximum clinical effect is not observed for 6 to 12 weeks. The thiazolidinediones are metabolized by the liver and may be administered to patients with renal insufficiency, but these agents should not be used if there is active hepatic disease or if there are significant elevations of serum liver transaminases.

Regular monitoring of liver funtion should be instituted in patients receiving thiazolidinediones. Thiazolidinediones also have been reported to cause anemia, weight gain, edema, and plasma volume expansion. These drugs generally are not indicated for patients with New York Heart Association class 3 and 4 heart failure.

Rosiglitazone and pioglitazone can lower hemoglobin $A_{1c}$ levels by 1.0% to 1.5% in patients with type 2 DM. These drugs can be combined with insulin or other classes of oral glucose-lowering agents. The thiazolidinediones tend to lower triglycerides (10% to 20%) but increase both HDL (up to 19%) and LDL (up to 12%) cholesterol. The increased LDL has been reported to reflect a change in particle size from a dense to a more buoyant, less atherogenic compound.

Both available thiazolidinediones are metabolized by cytochrome P450 enzymes in the liver. Rosiglitazone is metabolized by CYP2C8 and pioglitazone by CYP3A4 and CYP2C8. Metabolism by these hepatic enzymes provides the potential for interactions with other classes of drugs that are metabolized *via* these pathways. To date, no clinically significant interactions have been identified between the available thiazolidinediones and other drug classes, but further studies are in progress.

## α-Glucosidase Inhibitors

α-Glucosidase inhibitors reduce intestinal absorption of starch, dextrin, and disaccharides by inhibiting the action of intestinal brush border α-glucosidase. Inhibition of this enzyme slows the absorption of carbohydrates; the postprandial rise in plasma glucose is blunted in both normal and diabetic subjects.

*Acarbose* (PRECOSE), an oligosaccharide of microbial origin, and *miglitol* (GLYSET), a desoxynojirimycin derivative, also competitively inhibit glucoamylase and sucrase but have weak effects on pancreatic α-amylase. They reduce postprandial plasma glucose levels in type 1 DM and type 2 DM subjects. α-Glucosidase inhibitors can have profound effects on hemoglobin $A_{1c}$ levels in severely

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Goodman and Gilman's THE PHARMACOLOGICAL BASIS OF THERAPEUTICS, 10/e



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# Metformin modulates insulin post-receptor signaling transduction in chronically insulin-treated Hep G2 cells

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**KEY WORDS** metformin; insulin receptors; signal transduction

## ABSTRACT

**AIM:** To study the effect of chronic insulin treatment on insulin post-receptor signaling transduction and whether the effects of metformin are transmitted throughout the cascade of insulin signaling intermediates in a human hepatoma cell line (Hep G2). **METHODS:** Hep G2 cells were incubated in serum free media containing either insulin 100 nmol/L or insulin 100 nmol/L plus different concentrations (0.01-10 mmol/L) of metformin for 16 h and then were stimulated with insulin 100 nmol/L for 1 min. **RESULTS:** Chronic treatment of insulin 100 nmol/L induced a significant reduction in the phosphorylation and protein expression of IRß, IRS1 and IRS2, which therefore resulted in a downregulation of association of PI3K with IRS. Therapeutic concentrations (0.01-0.1 mmol/L) of metformin prevented the changes induced by chronic insulin treatment in these post-receptor components of insulin signaling pathway. Tyrosine phosphorylation of IRβ, IRS1, and IRS2 was increased by 2.7 fold ($P<0.01$), 6.8 fold ($P<0.01$), and 2.3 fold ($P<0.01$) of chronically insulin-treated cells alone, respectively, after metformin 0.1 mmol/L was added. The association of p85 with IRS1 and IRS2 was also increased from 34 % to 86 % ($P<0.01$) and from 30 % to 92 % ($P<0.01$), respectively. In contrast, metformin in pharmacological concentration (1-10 mmol/L) further inhibited tyrosine phosphorylation of IRβ, IRS1, IRS2 and the interaction of PI3K with IRS. The association of IRS1 with p85 was further decreased by 58 % ($P>0.05$) and of IRS2 by 30 % ($P<0.05$). **CONCLUSION:** Chronic insulin exposure of Hep G2 cells induces the downregulation of insulin signal transduction via PI3K pathway. The effect of metformin on insulin signaling transduction represent a primary mechanism of metformin action in insulin resistant state.

## INTRODUCTION

Metformin is an antihyperglycemic drug that treats insulin resistance and it has become a first-line choice of type 2 diabetes therapy. In recent years, research into the pharmacodynamic properties of metformin and their clinical implications has resulted in a renewal of interesting[1]. The liver is not only a key tissue for glucose metabolism, but also a major site of metformin action. Recently, the importance of hepatic insulin resistance in glucose homeostasis has been emphasized[2]. In hepatocytes, insulin resistance can result from impaired signaling downstream of the insulin receptor[3]. Antihyperglycemic effect of metformin is mainly a consequence of reduced hepatic glucose output.

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However, the molecular basis of metformin effect on hepatocytes is largely unknown, involving the possible effects of metformin on insulin signaling tranduction, for instance, metformin could directly or indirectly influence protein-protein interactions within the signaling cascade.

Chronic hyperinsulinsm can induce insulin resistance[4]. Therefore, in the present study, we set up an insulin resistant cell model, with the Hep G2 cells chronic exposure to high concentration of insulin. The aim is: (1) to examine the changes in insulin signaling transduction after chronic insulin treatment. (2) to determine whether the antihyperglycemic action of metformin in hepatocytes is associated with effects on signaling protein expression and phosphorylation.

## MATERIALS AND METHODS

**Chemicals** RPMI-1640 medium and fetal bovine serum (FBS) were obtained from Gibco BRL (Karlsruhe, Germany). Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were obtained from Bio-Rad (Hercules, CA). Rabbit polyclonal anti-insulin receptor β-subunit (IRβ), anti-insulin receptor substrate 1 (IRS1), anti-insulin receptor substrate 2 (IRS2), and anti-p85 subunit of phosphatidylinositol 3-kinase (PI3K) antibodies used for Western blotting were purchased from UBI (Lake Placid, NY). Anti-biotin and mouse monoclonal anti-phosphotyrosine (PY) antibody were from New England Biolabs (Frankfurt am Main, Germany). Protein A and protein G sepharose and BCA-assay reagents were purchased from Pierce (Hamburg, Germany). HRPO-conjugated anti-mouse and anti-rabbit antibodies, reagents for ECL were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Human insulin (Actrapid) was obtained from Novo Nordisk (Deisenhofen, Germany). Metformin, aprotinin, pepstatin, leupeptin, and other reagents were from Sigma (Steinheim, Germany).

**Cell culture** Hep G2 human hepatoma cells were grown to confluence in 10-cm plastic culture dishes containing 10 mL RPMI-1640 medium supplemented with 10 % fetal bovine serum, HEPES 25 mmol/L (pH 7.4), 1 % penicillin /streptomycin and glutamine 25 mmol/L. The cells were incubated at 37 °C in a humidified atmosphere of 5 % $CO_2$ and 95 % air.

Confluent Hep G2 cells were incubated in serum free media including either insulin 100 nmol/L or insulin 100 nmol/L plus metformin 0.01-10 mmol/L for 16 h and then were stimulated with insulin 100 nmol/L for 1 min, as specified in the results section. Cells under basal conditions were used as controls.

**Immunoprecipitation and Western blotting** Phosphorylated proteins of IRβ, IRS1, IRS2 and interaction of PI3K with IRS were determined by immunoprecipitation assay. protein lysates 1000 μg were subjected to immunoprecipitstion using corresponding specific antibodies (anti-IRβ, anti-IRS1, anti-IRS2) and incubation at 4 °C for 2 h. Protein-antibody complexes were conjugated with protein A and Protein G for another 1 h. Precipitates were take up in 30 μL sample buffer containing Tris 62.5 mmol/L, dithiothreitol (DTT) 100 mmol/L, 10 % glycerol, 2 % SDS, 0.01 % bromphenolblue and denatured at 95 °C for 10 min. Protein samples were subjected to 7.5 % SDS-PAGE. After SDS-PAGE, electrotransfer of protein from the gel to nitrocellulose membranes was performed by Western blotting. Subsequently, nitrocellulose filters were incubates at 4 °C overnight with one of the following antibodies (1st antibody) at a concentration of 1 mg/L: anti-PY, anti-IRβ, anti-IRS1, anti-IRS2 or anti-p85, each of those suspended in 2.5 % non-fat dried milk. Blots were then incubated with 1:3000 HRPO conjugate (2nd antibody) at room temperature (28 °C) for 1 h. Immunoreactive bands were determined with the ECL detected reagents.

**Immunoblotting** Relative protein levels of IRβ, IRS-1, IRS-2, and p85 were determined by Western-immunoblotting in total cell lysates. Similar size aliquots of sample (150 μg) were processed for 7.5 % SDS-PAGE and proteins were separated by electrophoresis. Immunoblotting was performed as described above.

**Statistical analysis** Relative amounts of immunoreactive proteins were quantitated by Image Quant scanning densitometry. Experimental results were expressed as mean±SD. Statistical analysis was performed by Student's *t*-test. Statistical significance was assessed at $P<0.05$.

## RESULTS

**Effect of metformin on protein levels and phosphorylation of signaling proteins following chronic insulin treatment** In the basal state, no or little tyrosine phosphorylation of IRβ, IRS1, and IRS2 was detectable. Acute stimulation with insulin for 1 min resulted in an extensive phosphorylation on tyrosine

residues of signaling proteins. After cells were treated with insulin 100 nmol/L for 16 h, insulin-induced autophosphorylations of IRβ, IRS1, and IRS2 were significantly decreased. By comparison with control cells, tyrosine phosphorylation of IRβ was decreased to 22 % ($P<0.01$) of control level. For IRS1 an even more significant reduction in insulin-induced phosphorylation was observed, as anti-PY detectable protein was reduced to 15 % ($P<0.01$). Similar to the changes in IRS1, tyrosine phosphorylation of IRS2 was decreased to 22 % ($P<0.01$) of control levels (Fig 1).

Therapeutic concentrations of metformin 0.01 mmol/L and 0.1 mmol/L reversed the reduction of tyrosine phosphorylation of insulin signaling protein induced by chronic insulin treatment. Tyrosine phosphorylation of IRβ was increased by 2.3 fold ($P<0.01$) and 2.7 fold ($P<0.01$) of chronically insulin treated cells alone. Tyrosine phosphorylation of IRS1 was increased by 6.4 fold ($P<0.01$) and 6.8 fold ($P<0.01$), and phosphorylation of IRS2 was increased 2.8 fold ($P<0.01$) and 2.3 fold ($P<0.05$) after cells were preincubated with insulin 100 nmol/L for 16 h in the simultaneous presence of metformin 0.01 mmol/L and 0.1 mmol/L, respectively.

When the concentration of metformin was further increased to pharmacological doses (1-10 mmol/L), the effect of metformin on insulin signal transduction became inhibitory. Tyrosine phosphorylation of IRβ was further decreased to 3 % ($P<0.01$) of control levels. Quite similar observations were made for IRS1 and IRS2. When metformin 10 mmol/L was added, IRS1 tyrosine phosphorylation was further decreased to only 4 % ($P<0.01$) and IRS2 phosphorylation to 11 % ($P<0.01$) of control levels (Fig 1).

There were also significant changes in protein expression levels of IRβ, IRS1, and IRS2 following insulin 100 nmol/L chronic treatment. Protein level of IRβ was decreased to 42 % ($P<0.01$) of control levels, IRS1 was decreased to 63 % ($P<0.01$) and IRS2 was decreased to 47 % ($P<0.05$) compared to controls. However, this chronic hyperinsulinism induced reduction of protein levels was reversed after cells were preincubated with different concentrations of metformin. Level of IRβ was increased by 1.1 fold ($P<0.01$) in the presence of metformin 0.01 mmol/L and 1.6 fold ($P<0.01$) in the presence of metformin 10 mmol/L. Levels of IRS1 and IRS2 did not significantly changed following physiological concentration of metformin treatment and increased by 43 % ($P<0.01$) and 112 %

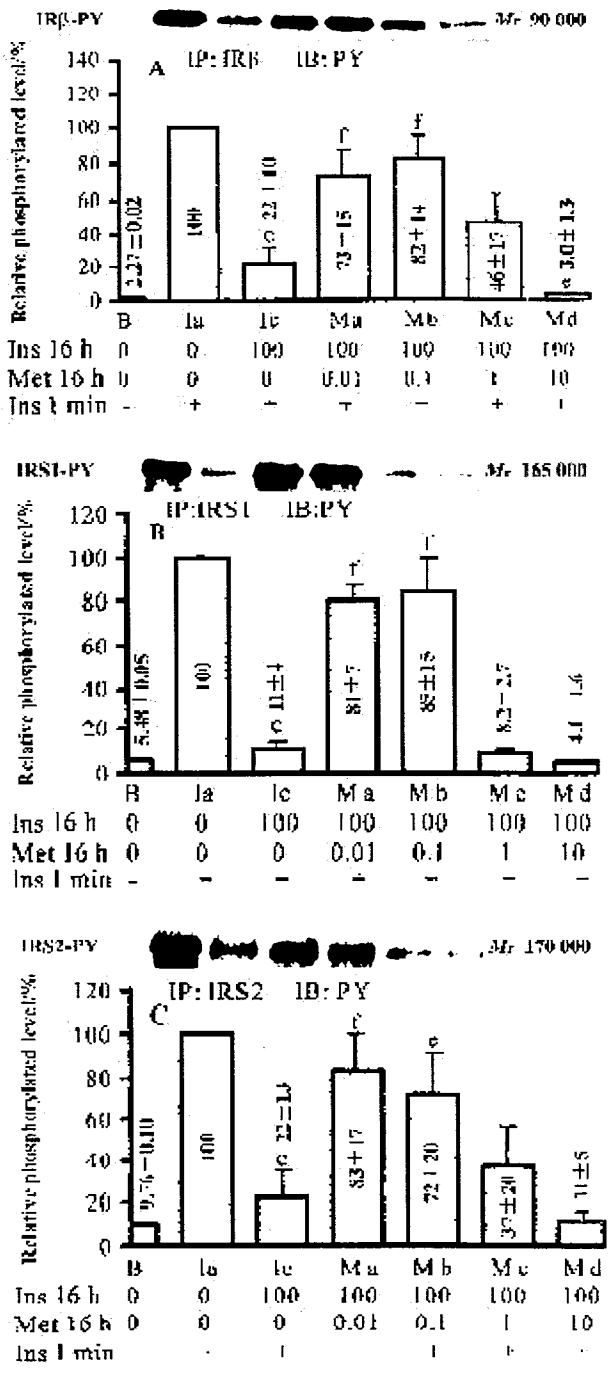


**Fig 1. Effects of metformin on tyrosine phosphorylation of IRβ (A), IRS1 (B), and IRS2 (C) after chronic insulin treatment. Hep G2 cells were treated either without insulin (Ia), or with insulin 100 nmol/L (Ic) or with insulin 100 nmol/L and metformin 0.01, 0.1, 1, 10 mmol/L (Ma, Mb, Mc, Md) for 16 h and then were stimulated with insulin 100 nmol/L for 1 min, and cells cultured without these drugs were used to demonstrate the basal level (B). A typical blot for scanning densitometry by Image Quant (Molecular Dynamics) is shown above. $n$=4. Mean±SD. They are expressed as relative to Ia values, which were set at 100 %. $^cP<0.01$ *vs* Ia. $^eP<0.05$, $^fP<0.01$ *vs* Ic. IP, immunoprecipitation; IB, immunoblot; Met, metformin; Ins, insulin.**

($P<0.01$) by exposure to metformin 10 mmol/L (Tab 1).

**Tab 1. Effects of metformin on protein levels of insulin signaling molecules after chronic insulin treatment. $n$=4. Mean±SD. $^cP<0.01$ *vs* control. $^eP<0.05$, $^fP<0.01$ *vs* Ic.**

| | IRβ | IRS1 | IRS2 | p85 |
|---|---|---|---|---|
| Control | 0.167±0.012 | 0.35±0.03 | 0.241±0.010 | 0.38±0.03 |
| Ia | 0.18±0.06 | 0.34±0.06 | 0.238±0.017 | 0.39±0.05 |
| Ic | 0.07±0.03$^c$ | 0.217±0.021$^c$ | 0.107±0.017$^c$ | 0.36±0.05 |
| Ma | 0.15±0.05$^f$ | 0.27±0.05$^e$ | 0.173±0.023 | 0.37±0.04 |
| Mb | 0.13±0.05$^e$ | 0.26±0.04$^e$ | 0.152±0.017 | 0.35±0.06 |
| Mc | 0.21±0.07$^f$ | 0.29±0.05$^e$ | 0.134±0.021 | 0.37±0.06 |
| Md | 0.19±0.04$^f$ | 0.31±0.03$^f$ | 0.24±0.05$^f$ | 0.38±0.05 |

HepG2 cells were treated either without addition of insulin (Ia), or with insulin 100 nmol/L (Ic) or with insulin 100 nmol/L plus metformin 0.01, 0.1, 1, 10 mmol/L (Ma, Mb, Mc, Md) for 16 h and then stimulated with insulin 100 nmol/L for 1 min, cells cultured without these drugs were used to demonstrate the basal control. The data are OD values of Scanning densitometry by Image Quant (Molecular Dynamics).

**Effect of metformin on the interaction of IRS1 and IRS2 with PI3K following chronic insulin treatment** To determine the effect of reduced IRS1 and IRS2 expression and phosphorylation after chronic insulin treatment on their interaction with the p85 subunit of PI3K, blots with immunopresipitates for IRS1 and IRS2 were immunoblotted with anti-p85 antibodies. As described above, cells pretreated in the absence of insulin responded to an acute maximal insulin stimulation with an increase in IRS-associated p85 of more than 10-fold. In contrast, after the cells were exposed to insulin 100 nmol/L for 16 h, immunodetectable p85 was significantly decreased, which was consistent with changes in phosphorylation of IRS1 and IRS2. However, in the presence of metformin 0.01 mmol/L, these effects of chronic insulin treatment on the association of IRS with p85 were reserved. Immunode-tectable p85 was increased from 34 % to 86 % ($P<0.01$) of control levels in anti-IRS1 immunoprecipitates and from 30 % to 92 % ($P<0.01$) in anti-IRS2 immunoprecipitates. When the dose of metformin was further increased to the pharmacological concentration 10 mmol/L, association of IRS1 with p85 was further decreased by 58 % ($P>0.05$) and of IRS2 by 30 % ($P<0.05$) of chronic treatment with insulin 100 nmol/L alone (Fig 2).

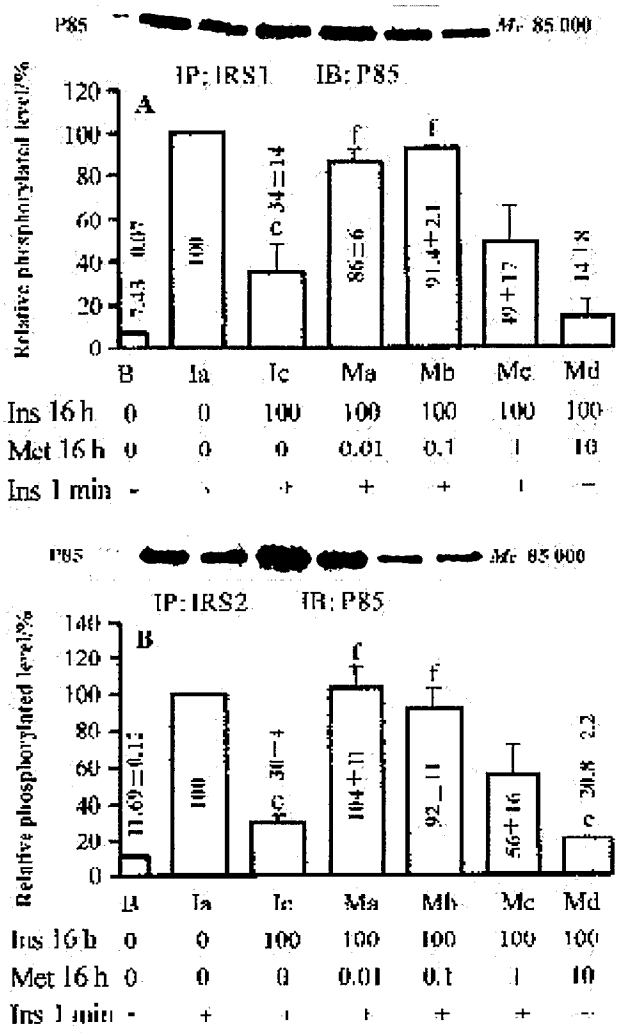


**Fig 2. Effects of metformin on association of p85 subunit of PI3K with IRS1 (A) and IRS2 (B) after chronic insulin treatment. HepG2 cells were treated either without insulin (Ia), or with insulin 100 nmol/L (Ic) or with insulin 100 nmol/L and metformin 0.01, 0.1, 1, 10 mmol/L (Ma, Mb, Mc, Md) for 16 h and then were stimulated with insulin 100 nmol/L for 1 min, and cells cultured without these drugs were used to demonstrate the basal level (B). A typical blot for scanning densitometry by Image Quant (Molecular Dynamics) is shown above. $n$=4. Mean±SD. They are expressed as relative to Ia values, which were set at 100 %. $^cP<0.01$ *vs* Ia. $^eP<0.05$, $^fP<0.01$ *vs* Ic. IP, immunoprecipitation; IB, immunoblot; Met, metformin; Ins, insulin.**

The protein expression of p85 was not significantly changed, either therapeutic or pharmacological concentrations of metformin added to the media (Tab 1).

## DISCUSSION

Chronic hyperinsulinism can induce insulin resistance[4]. Cells cultured exposured to the high concentrations of insulin is an established model to induce

insulin resistance *in vitro*[4,5]. Thereby, in the present study, we first set up an insulin resistant model by using chronic treatment of Hep G2 cells with high doses of insulin. Such *in vitro* allows direct assessment of the effect of an additional treatment on the biological response to insulin stimulation. Since metformin exerts its beneficiary antihyperglycemic effect primarily in liver, this should also involve an alteration of the insulin resistant state in this organ. Therefore, it appeared interesting to focus on determining relevant molecules which transmit the effects of metformin within the insulin signaling cascade in a liver cell model system, in which insulin resistance had been induced. The data described here have suggested that impaired insulin signal transduction linked to IRβ, IRS1, IRS2, and PI3K is associated with insulin resistance, which was caused by chronic insulin treatment.

Metformin could interact with insulin at many potential steps, including the increased binding of insulin to its receptor[6], the intensified of insulin receptor tyrosine kinase activity[7-9], the elevated inositol-1,4,5-trisphosphate production, the augmented glycogen synthesis, and the inhibition of PEPCK as a key enzyme of gluconeogenesis[10]. In present experiment, the effect of chronic insulin treatment can be reversed by metformin. It suggests that metformin's action site is most likely located at an early post-receptor level and may directly or indirectly interact with the intracellular insulin signaling cascade.

Up to now, there were only a few reports regarding the effect of metformin on intracellular insulin signaling system and there has been a controversy in different experiments. Metformin was reported to increase insulin signaling transduction in cholesterol-treated Hep G2 cells[11] and to reverse chronic insulin effects on insulin signaling in rat adipocytes[5]. However, metformin treatment had no effect on insulin signaling cascade in human adipoctey[12] and skeletal muscle[13]. These different observations may be due to difference of tissues and cultured conditions. In the present study, therapeutic doses of metformin have reversed the reduction in phosphorylation of IRβ , IRS1, and IRS2 induced by chronic insulin treatment. Namely, through elevation of tyrosine phosphorylation of the insulin receptor metformin can increase further tyrosine phosphorylation of IRS1 and IRS2, as well as the association of IRS1 and IRS2 with PI3K. PI3K has been shown to play a critical role in many insulin-regulated metabolic processes, including stimulation of glucose transport, activation of glycogen synthase, and inhibition of PEPCK as the key enzyme of gluconeogenesis. Since the effect of metformin on insulin signaling processes was observed at concentrations reached in the serum of metformin-treated patients, these data are somewhat suggestive for the actual situation in humans. Thus, the primary mechanism of metformin's action to restore insulin sensitivity in hepatocytes might be related to the increased insulin post-receptor signal transduction linked to tyrosine phosphorylation of IRβ, IRS1 and IRS2 and the activation of PI3K.

In contrast to the effect of metformin at therapeutical concentrations, pharmacological concentrations of metformin inhibit further phosphorylation of signaling proteins and association of IRS with PI3K. These inhibitory effects may reflect the fact that higher metformin concentrations inhibit insulin action[9]. It should be noted that the changes in tyrosine phosphorylation associated with metformin treatment were not parallel to the alterations of protein expression level, which were increased in the presence of pharmacological concentrations of metformin. This suggests that effect of metformin on tyrosine phosphorylation of signaling protein could not be explained by the changes in the level of protein expression. Whether inhibitory effects of pharmacological concentrations of metformin are correlated with either the alteration in insulin receptor, IRS1 and IRS2 serine phosphorylation, or the direct action of metformin on insulin signal transduction or other regulatory event, remains to be investigated in future studies.

In conclusion, the present data suggest that chronic insulin exposure of Hep G2 cells results in down-regulation of insulin signal transduction via PI3K pathway. Therapeutic concentrations of metformin can reverse the effect of chronic hyperinsulinism on expression and activation of insulin signaling molecules, and pharmacological concentrations of metformin inhibit insulin signal transduction. The effect of metformin on insulin signal transduction represents a primary mechanism of metformin action in insulin resistant state.

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SPECIAL REPORT

# The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1)

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The endogenous cannabinoid anandamide was identified as an agonist for the recombinant human VR1 (hVR1) by screening a large array of bioactive substances using a FLIPR-based calcium assay. Further electrophysiological studies showed that anandamide (10 or 100 $\mu$M) and capsaicin (1 $\mu$M) produced similar inward currents in hVR1 transfected, but not in parental, HEK293 cells. These currents were abolished by capsazepine (1 $\mu$M). In the FLIPR anandamide and capsaicin were full agonists at hVR1, with $pEC_{50}$ values of $5.94 \pm 0.06$ ($n=5$) and $7.13 \pm 0.11$ ($n=8$) respectively. The response to anandamide was inhibited by capsazepine ($pK_B$ of $7.40 \pm 0.02$, $n=6$), but not by the cannabinoid receptor antagonists AM630 or AM281. Furthermore, pretreatment with capsaicin desensitized the anandamide-induced calcium response and vice versa. In conclusion, this study has demonstrated for the first time that anandamide acts as a full agonist at the human VR1.
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**Keywords:** Vanilloid; capsaicin; calcium; anandamide; cannabinoid; FLIPR; nociception

**Abbreviations:** AM281, (1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide); AM630, (6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone);$[Ca^{2+}]_i$ intracellular calcium concentration; CP-55,940, ((−)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol); DRG, dorsal root ganglion; FI, fluorescence intensity; FLIPR, fluorometric imaging plate reader; hVR1, human VR1; MEM, minimum essential medium; VR1, vanilloid receptor; WIN-55,212-2, ((R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone)

**Introduction** The vanilloid receptor (VR1) has recently been cloned (Caterina *et al.*, 1997) and is a ligand-gated ion channel which plays an important role in nociception (Szallasi & Blumberg, 1999). Although the pungent plant extract capsaicin activates VR1 (Caterina *et al.*, 1997), the endogenous mammalian ligand remains to be identified (Szallasi & Blumberg, 1999).

We screened a collection of over 1000 bioactive substances for activity at the recombinant VR1, and identified the endogenous cannabinoid anandamide (Devane *et al.*, 1992) as an agonist at this receptor. This finding was of particular interest for several reasons. Firstly, the structure of anandamide (Pertwee, 1997) bears a striking similarity to several vanilloids (Szallasi & Blumberg, 1999), most notably olvanil (DiMarzo *et al.*, 1998). Secondly, anandamide has vasodilatory effects (Pertwee, 1997), which are not mediated by the cannabinoid receptors (White & Hiley, 1998). Thirdly, olvanil has been shown to inhibit the anandamide transporter (DiMarzo *et al.*, 1998; Beltramo & Piomelli, 1999). In addition anandamide has recently been reported to act as a partial agonist at the rat VR1 receptor (Zygmunt *et al.*, 1999).

Therefore, in the present study the effects of anandamide, and other cannabinoid ligands (Pertwee, 1997), on HEK293 cells stably expressing the hVR1 have been characterized using electrophysiology and a FLIPR-based calcium influx assay. This study has shown for the first time that anandamide acts as a full agonist at the human VR1.

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**Methods** *Cloning and expression of VR1 receptors in HEK293 cells* Human VR1 cDNA was identified using the published rat VR1 sequence (GenBank accession AF029310) to search public nucleotide databases. Expressed sequence tag T48002 was identified and its sequence extended by rapid amplification of the cDNA ends using cDNA templates from a number of tissue sources. The full cDNA was amplified from brain cDNA, inserted into the expression vector pcDNA3.1, double strand sequenced, and stably expressed in HEK293 cells. Rat VR1 cDNA was amplified from rat DRG cDNA and similarly expressed in HEK293 cells.

*Cell culture* hVR1-HEK293 cells were grown as monolayers in minimum essential medium (MEM) supplemented with non-essential amino acids, 10% foetal calf serum, and 0.2 mM L-glutamine, and maintained under 95%/5% $O_2/CO_2$ at 37°C. Cells were passaged every 3–4 days and the highest passage number used was 20. Dissociated rat neonatal DRG cultures were prepared as described by Skaper *et al.* (1990).

*Electrophysiological studies* Cells were plated and cultured on glass coverslips at 26,000 cells $cm^{-2}$ and whole-cell voltage-clamp recordings were performed at room temperature (20–24°C), using standard methods. The extracellular solution consisted of (mM): NaCl, 130; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; Glucose, 30; HEPES-NaOH, 25, pH 7.3. For anandamide application this solution was supplemented with 0.2% lipid free bovine serum albumin. Patch pipettes of resistance 2–5 MΩ were fabricated on a Sutter Instruments P-87 electrode puller and were filled with the following solution (mM): CsCl, 140; $MgCl_2$, 4; EGTA, 10; HEPES-CsOH, 10, pH 7.3. All recordings were made from single, well isolated, phase bright

cells. Currents were recorded at a holding potential of −70 mV using a Axopatch 200B amplifier. Data acquisition and analysis were performed using the pClamp7 software suite. Drug applications were affected with an automated fast-switching solution exchange device (Warner Instruments SF-77B; time for solution exchange ~30 ms).

*Measurements of $[Ca^{2+}]_i$ using the FLIPR* hVR1-HEK293 cells were seeded into black walled clear-base 96-well plates (Costar U.K.) at a density of 25,000 cells per well in MEM, supplemented as above, and cultured overnight. The cells were then incubated with MEM containing the cytoplasmic calcium indicator, Fluo-3AM (4 μM; Teflabs, Austin, TX, U.S.A.) at 25°C for 120 min. The cells were washed four times with, and finally cultured in, Tyrode's medium containing 0.2% BSA, before being incubated for 30 min at 25°C with either buffer alone (control) or buffer containing various antagonists. The plates were then placed into a FLIPR (Molecular Devices, U.K.) to monitor cell fluorescence ($\lambda_{EX}$=488 nM, $\lambda_{EM}$=540 nM) (Sullivan *et al.*, 1999) before and after the addition of various agonists.

*Data analysis* Responses were measured as peak fluorescence intensity (FI) minus basal FI, and where appropriate, were expressed as a percentage of a maximum capsaicin-induced response. Data are expressed as mean±s.e.mean unless otherwise stated. Curve-fitting and parameter estimation were carried out using Graph Pad Prism 3.00 (GraphPad Software Inc., CA, U.S.A.). p$K_B$ values were generated from $IC_{50}$ curves for the antagonist vs a fixed $EC_{80}$ concentration of agonist using the Cheng-Prusoff equation.

*Materials* All cannabinoids were purchased from Tocris (Bristol, U.K.) and all other ligands were obtained from RBI (Natick, MA, U.S.A.). All cell culture media were obtained from Life Technologies (Paisley, U.K.).

**Results** Application of either anandamide (10 μM) or capsaicin (1 μM) produced inward currents in HEK293 cells stably transfected with the hVR1 receptor. Neither agent produced responses in parental HEK293 cells. The currents produced by both agents developed slowly with mean time constants of 3.78±0.93 s (1 μM capsaicin, $n$=7) and 4.01±0.65 s (10 μM anandamide, $n$=9) reading peak amplitudes of 122±27 and 44±13 pA, respectively. The currents produced by either agonist exhibited substantial outward rectification and had similar interpolated reversal potentials (2.0±1.4 mV, anandamide; −1.6±1.2 mV, capsaicin; $n$=5). The steady-state currents produced by both anandamide and capsaicin could be completely blocked by subsequent co-application of 1 μM capsazepine (Figure 1A); this antagonism developed with mean time-constants of 941±205 ms ($n$=9) and 3406±930 ms ($n$=7), respectively ($P$<0.05, unpaired Student's $t$-test). In cultured rat DRG neurons anandamide (10 or 100 μM) produced capsazepine-sensitive inward currents in all capsaicin-responsive cells tested (Figure 1B,C). Even in response to 100 μM anandamide the amplitude of the currents recorded were smaller (~10–50%) than those generated by VR1 activation with a maximum concentration of capsaicin.

In the FLIPR, anandamide (100 pM–10 μM), like capsaicin (100 pM–10 μM), caused a concentration-dependent increase in $[Ca^{2+}]_i$ in hVR1-HEK293 cells (Figure 2), but was without effect in the non-transfected HEK293 cell-line (data not shown). Anandamide displayed a similar efficacy to capsaicin (Figure 2), but was markedly less potent ($pEC_{50}$ values of 5.94±0.06 and 7.13±0.11, respectively, at pH 7.4). Moreover, the anandamide- and capsaicin-induced $Ca^{2+}$ responses had indistinguishable kinetics in the FLIPR, with an initially rapid then slowing onset (peak ~30 s) followed by a gradually declining secondary phase.

The anandamide analogues methanandamide and palmitoylethanolamide also increased $[Ca^{2+}]_i$ in hVR1-HEK293 cells in a concentration-related manner, but were less potent

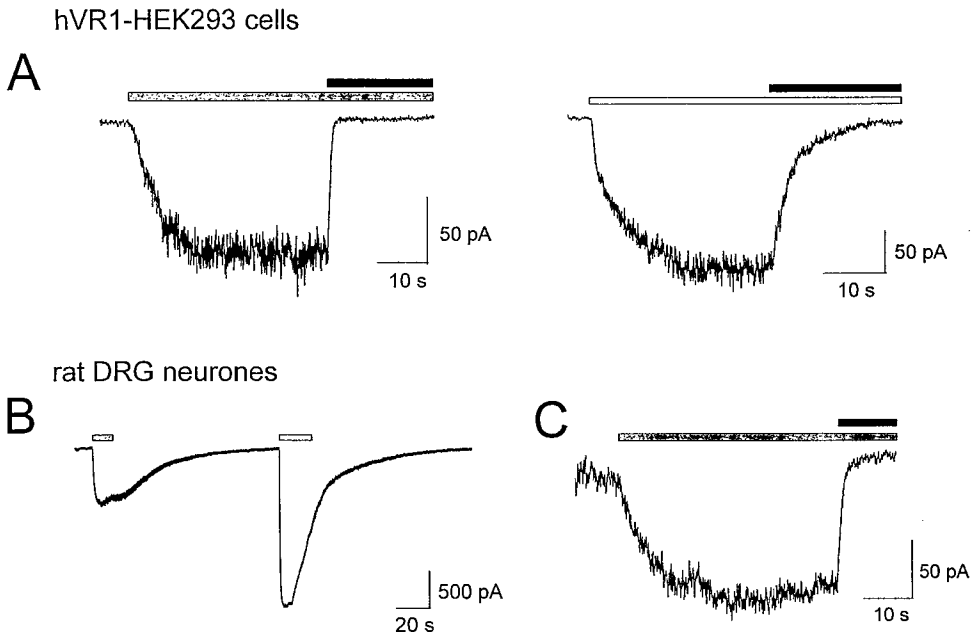


**Figure 1** Anandamide-gated currents in hVR1-HEK293 cells and rat DRG neurons are capsazepine sensitive. (A) Application of anandamide (10 μM; grey bar) or capsaicin (1 μM; open bar) led to the appearance of inward currents in cells voltage clamped at −70 mV. These currents were blocked completely by co-application of capsazepine (1 μM; solid bar). Data are representative traces, typical of $n$=7–9. (B) Application of anandamide (100 μM; shaded bar) led to the appearance of inward currents in DRG neurons voltage clamped at −70 mV. These cells were also shown to be sensitive to capsaicin (1 μM; open bar). (C) Anandamide-gated currents were blocked by capsazepine (1 μM; solid bar). Data are representative traces, typical of $n$=3.

than anandamide, only evoking ~40% responses at 10 μM (Figure 2 and data not shown, $n=3$). Like anandamide both these ligands were without effect in the non-transfected HEK293 cells. The synthetic cannabinoids CP-55,940 (((−)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol)) and WIN-55,212-2 (((R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone)) were without effect in parental or hVR1-expressing HEK293 cells.

Interestingly, lowering the pH of the experimental buffer from 7.4 to 6.4 enhanced the potency of capsaicin ($pEC_{50}$ of 7.13±0.11 at pH 7.4 and 7.86±0.18 at pH 6.4, $n=3$), but had no effect on the potency of anandamide ($pEC_{50}$ of 5.94±0.06 at pH 7.4 and 5.76±0.04 at pH 6.4, $n=3$), or any of the other cannabinoid ligands tested (Figure 2 and data not shown).

The competitive VR1 antagonist (Szallasi & Blumberg, 1999) capsazepine (100 pM–100 μM) inhibited both the anandamide (3 μM)- and capsaicin (100 nM)-induced $Ca^{2+}$ responses, with $pK_B$ values of 7.40±0.02 and 7.31±0.3 respectively ($n=6$). However, the cannabinoid receptor antagonists AM630 ((6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone)) and AM281 ((1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide)) (100 pM–10 μM) had no effect on either response (data not shown). Furthermore, pretreatment with 1 μM capsaicin for 30 min desensitized the response to 10 μM anandamide (16,271±789 vs 332±26 FI, $n=6$) and 10 μM anandamide desensitized the response to 100 nM capsaicin (15,283±1076 vs 1245±83 FI, $n=6$).

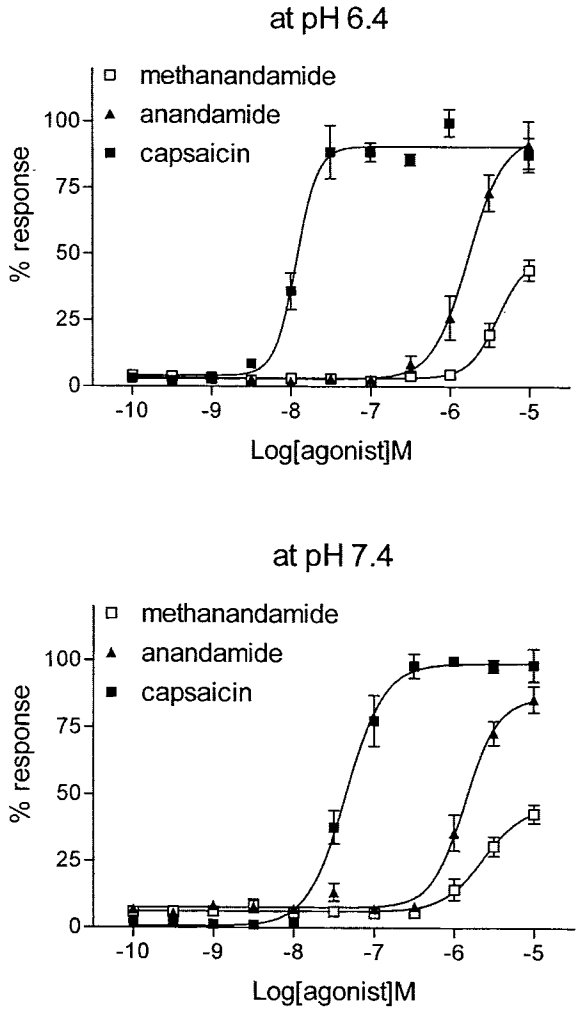


**Figure 2** Anandamide-induced $Ca^{2+}$ responses are concentration-dependent $[Ca^{2+}]_i$ was monitored using Fluo-3AM in hVR1-HEK293 cells before and after the addition of capsaicin (100 pM–10 μM), anandamide (100 pM–10 μM) or methanandamide (100 pM–10 μM) at pH 6.4 or 7.4. Responses were measured as peak increase in fluorescence minus basal, expressed relative to the maximum capsaicin response and are given as mean±s.e.mean, where $n=3-8$.

**Discussion** VR1 is a ligand-gated ion channel which plays an important role in nociceptive signalling (Szallasi & Blumberg, 1999). This receptor is activated by the plant extract capsaicin (Caterina *et al.*, 1997), but the identity of the endogenous mammalian ligand remains unclear (Szallasi & Blumberg, 1999). Screening a wide range of bioactive substances revealed that anandamide, an endogenous cannabinoid (Devane *et al.*, 1992), acted as an agonist at VR1. Anandamide displays a high structural similarity to the vanilloids, especially olvanil (DiMarzo *et al.*, 1998; Beltramo & Piomelli, 1999), and has recently been reported to activate rat VR1 (Zygmunt *et al.*, 1999). Therefore, the present study examined the pharmacology of anandamide at the recombinant human VR1 using electrophysiology and a FLIPR-based calcium assay, and has demonstrated for the first time that anandamide acts at human VR1 as a full agonist.

In the present study anandamide activated an inward current in hVR1-expressing, but not in parental, HEK293 cells. This current displayed similar kinetics to the capsaicin-induced current and was inhibited by capsazepine, a VR1 antagonist (Szallasi & Blumberg, 1999). Moreover, the anandamide- and capsaicin-induced currents had similar reversal potentials close to 0 mV, consistent with the gating of a non-selective ion channel and similar to findings for rat VR1 (Caterina *et al.*, 1997). Anandamide also produced similar capsazepine-sensitive inward currents in capsaicin-sensitive cultured rat DRG neurons. In both the rat DRG neurones and hVR1-HEK293 cells the peak amplitude of the anandamide-induced current was significantly smaller (<50%) than that of the capsaicin-induced current. Similar results have recently been reported for anandamide at the recombinant rat VR1 (Zygmunt *et al.*, 1999). Nevertheless, this apparent partial agonism probably reflects the technical difficulties in applying sufficiently high concentrations of such a lipophilic ligand in electrophysiological studies.

In the FLIPR anandamide and capsaicin both increased $[Ca^{2+}]_i$ in hVR1-expressing, but not non-transfected, HEK293 cells, and these responses displayed virtually identical kinetics. More importantly, the concentration-response relationship for anandamide was defined, and showed that anandamide was a full agonist compared to capsaicin. Capsaicin was more potent than anandamide, with an affinity consistent with that previously reported for rat VR1 (Caterina *et al.*, 1997). Indeed, the potency of anandamide at hVR1 was ~20 fold lower than its binding affinity (55 nM) at the cannabinoid receptor (Devane *et al.*, 1992), but was more consistent with the affinities (160–540 nM) reported from functional studies (Pertwee, 1997). Moreover, VR1 is structurally related to the transient receptor potential (TRP) channel family (Caterina *et al.*, 1997), and certain TRP channels are activated by other lipid messengers with similar potencies (Chyb *et al.*, 1999). Interestingly, the potency of capsaicin at hVR1 was enhanced by lowering the pH from 7.4 to 6.4, as reported for the rat VR1 (Caterina *et al.*, 1997). In contrast the potency of anandamide was unaffected by pH, suggesting that anandamide and capsaicin may either bind to different

sites on the human VR1 or gate the channel by different mechanisms.

Several lines of evidence demonstrate that the anandamide-induced $Ca^{2+}$ response is mediated by hVR1. Firstly, anandamide has no effect in parental HEK293 cells. Secondly, the anandamide-induced $Ca^{2+}$ response is inhibited by capsazepine, with a $pK_B$ value consistent with the affinity of capsazepine at VR1 (Szallasi & Blumberg, 1999). Thirdly, the cannabinoid receptor antagonists, AM630 and AM281 (Pertwee, 1997) had no effect on the anandamide-induced response. Fourthly, capsaicin causes a homologous desensitization of the anandamide-induced response, and vice versa. Finally, the synthetic cannabinoid agonists, CP-55,950 and WIN-55,212-2 (Pertwee, 1997) were inactive at hVR1. These synthetic cannabinoids also failed to elicit a response in rVR1-HEK293 cells (Zygmunt *et al.*, 1999). However, in the present study the putative endogenous peripheral cannabinoid receptor ligand, palmitoylethanolamide (Pertwee, 1997) also activated hVR1, albeit more weakly than anandamide, despite having been reported to be inactive at the rat VR1 (Zygmunt *et al.*, 1999). This demonstrates that other endogenous lipids can activate hVR1, suggesting possible roles for many different lipids in nociception. In conclusion, the present study has clearly demonstrated for the first time that the endogenous cannabinoid, anandamide acts as a full agonist at the human VR1.

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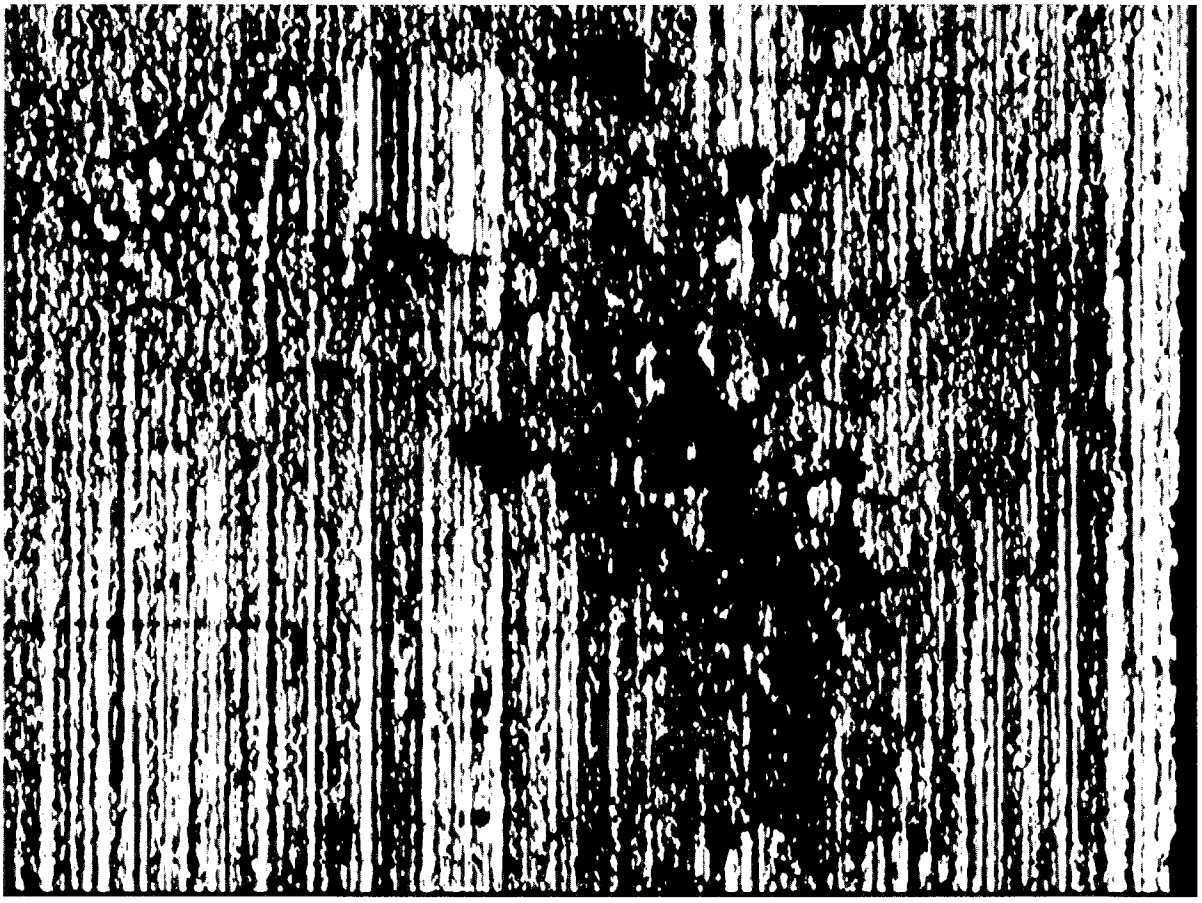
Exhibit 3A1



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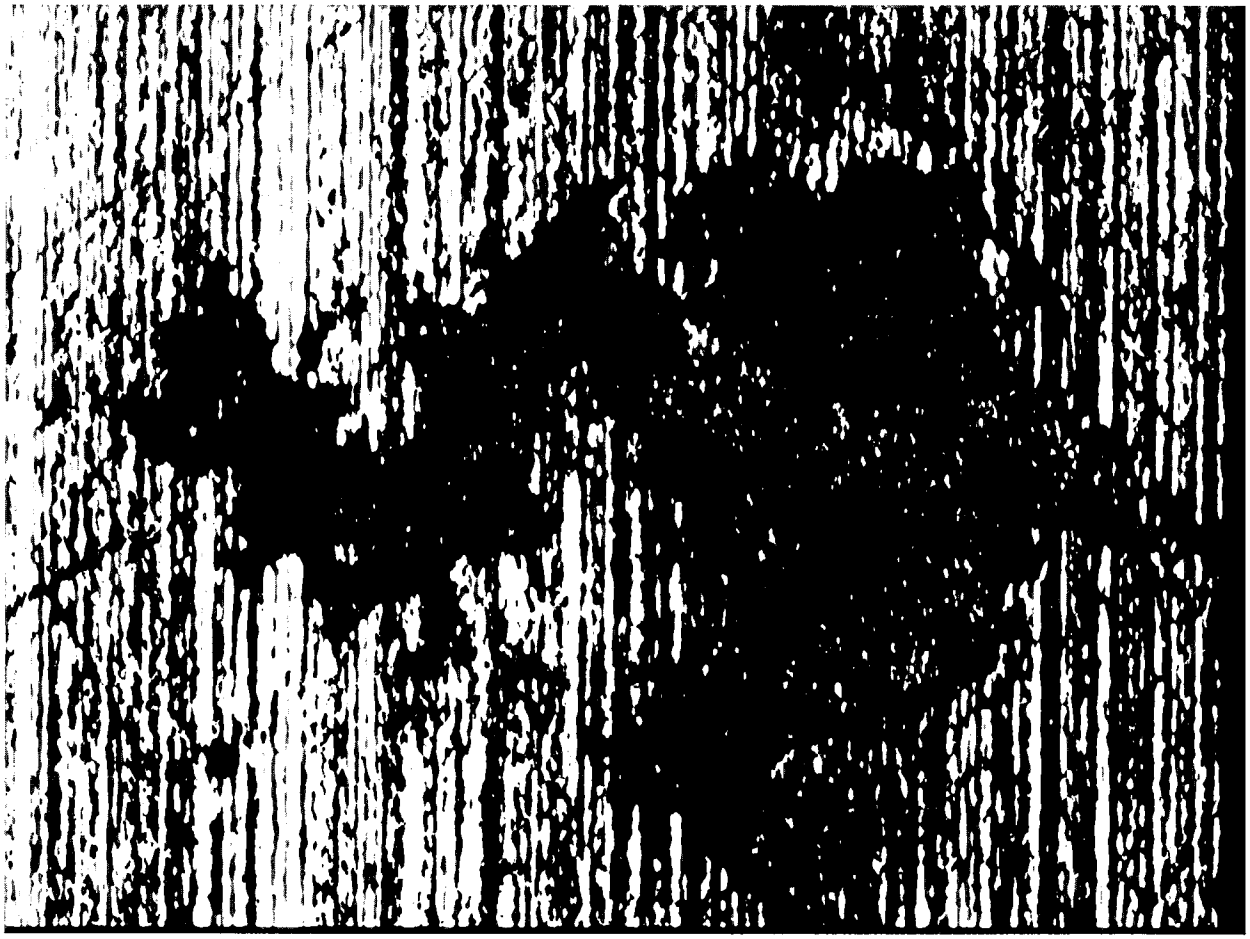

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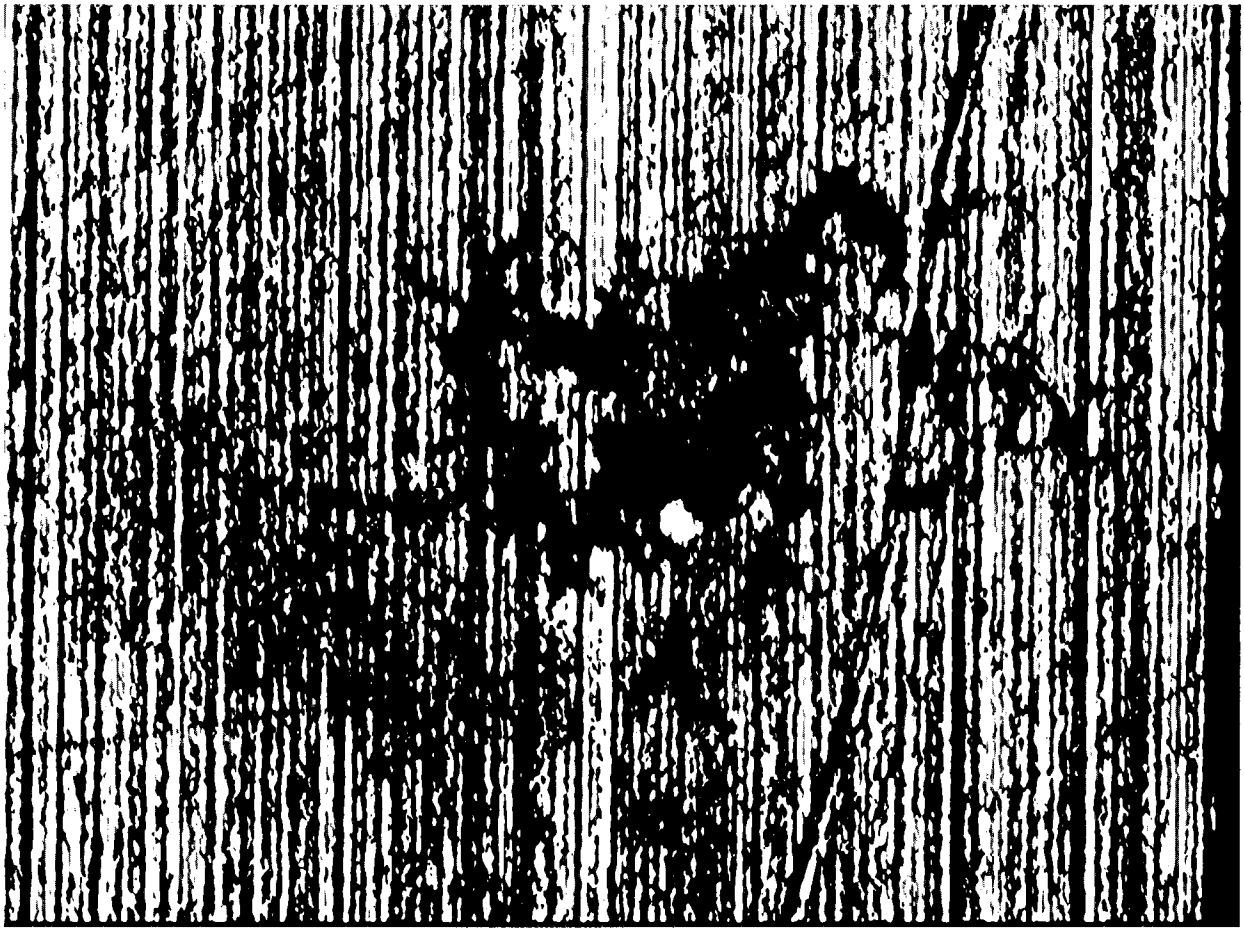
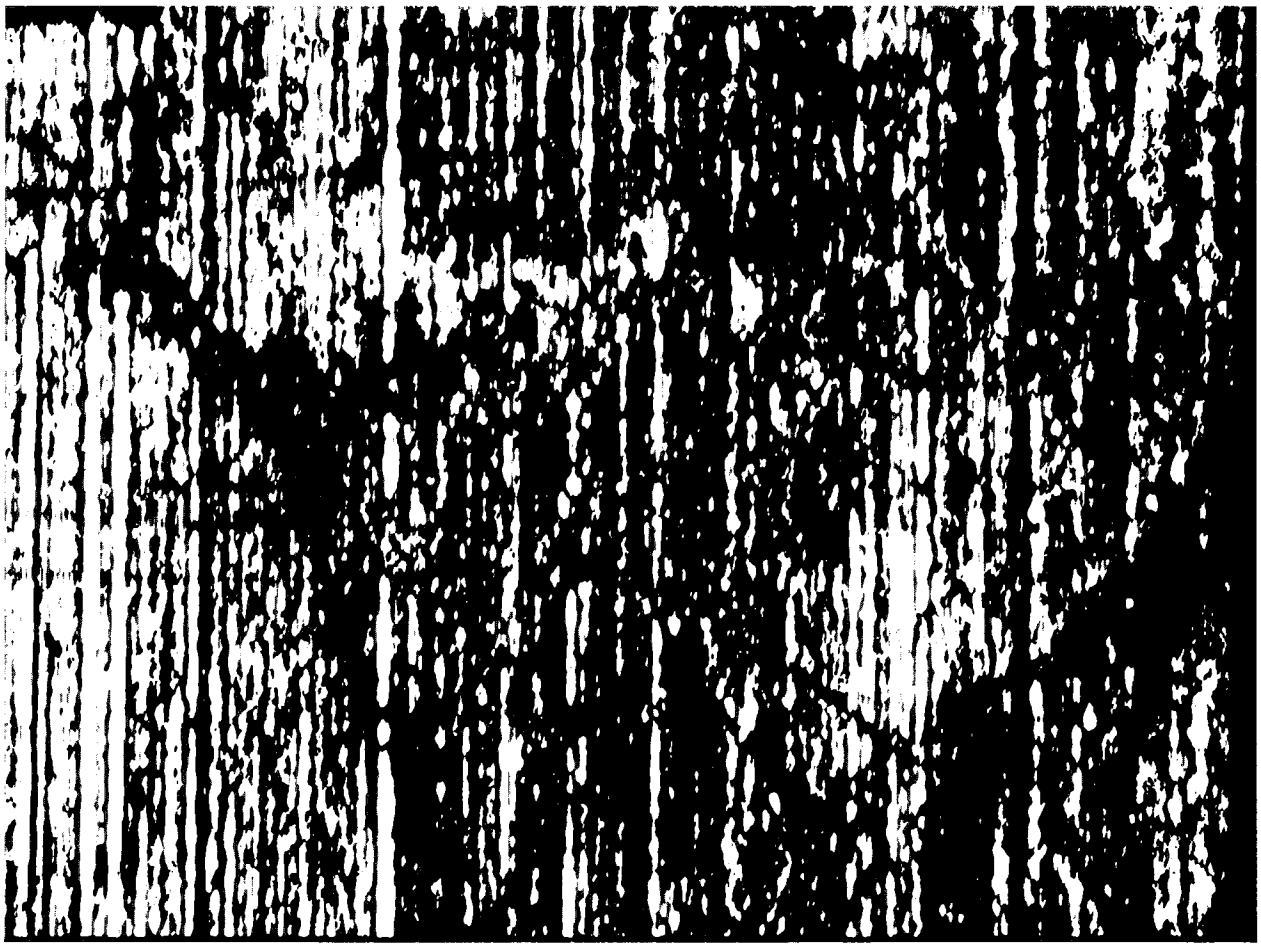


Exhibit 3C1a

Exhibit 3C1b

Exhibit 3C2a



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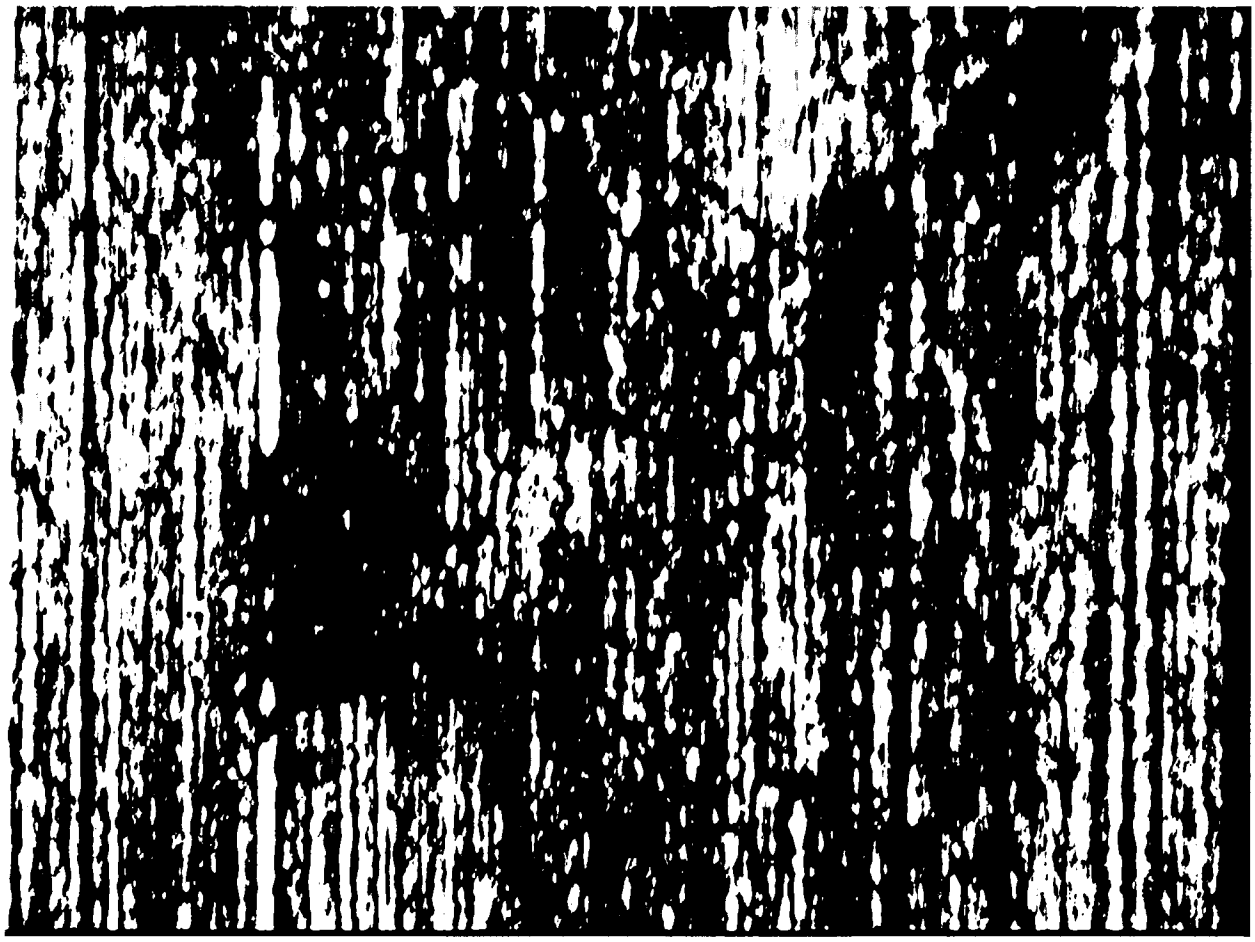

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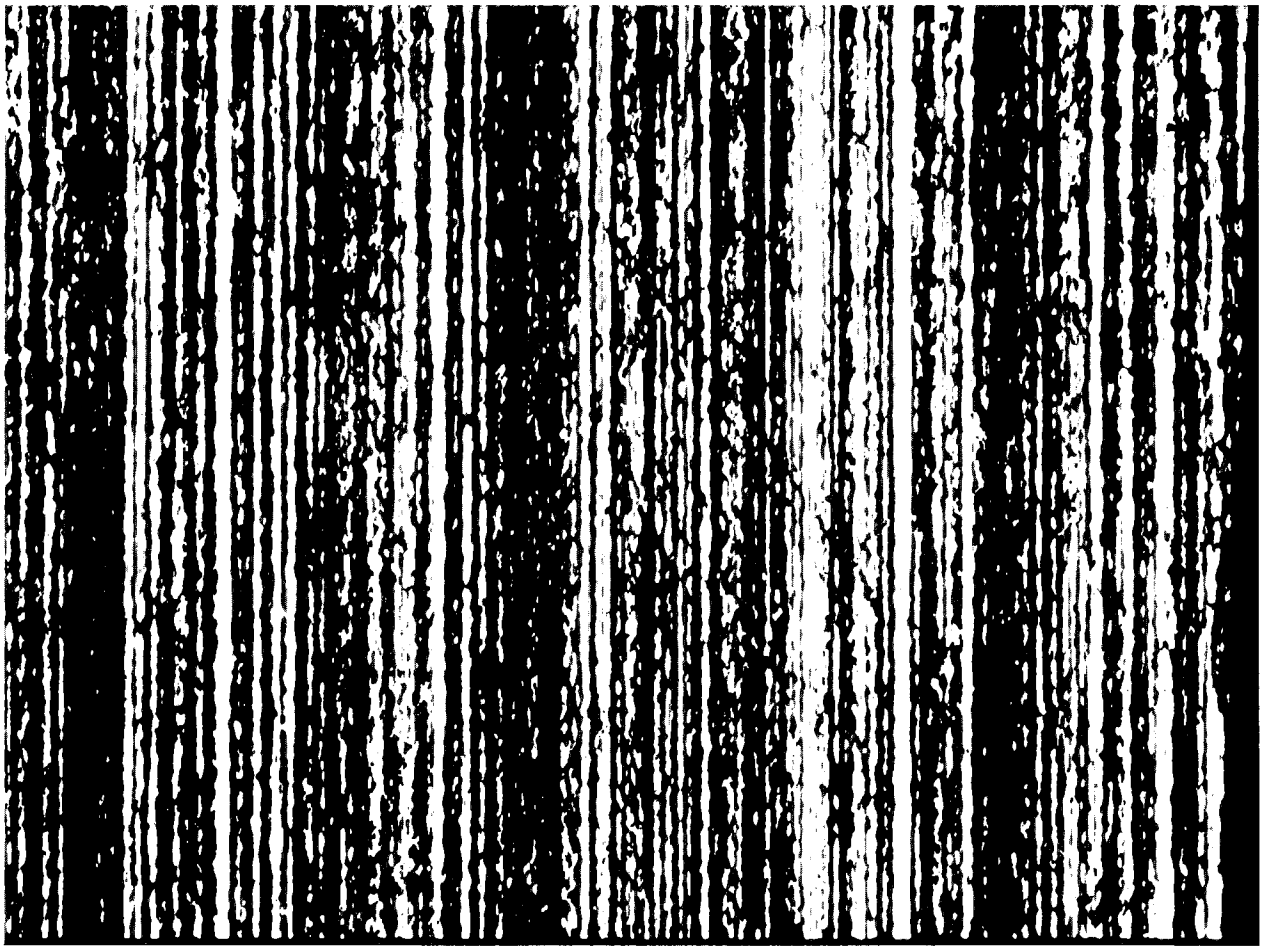

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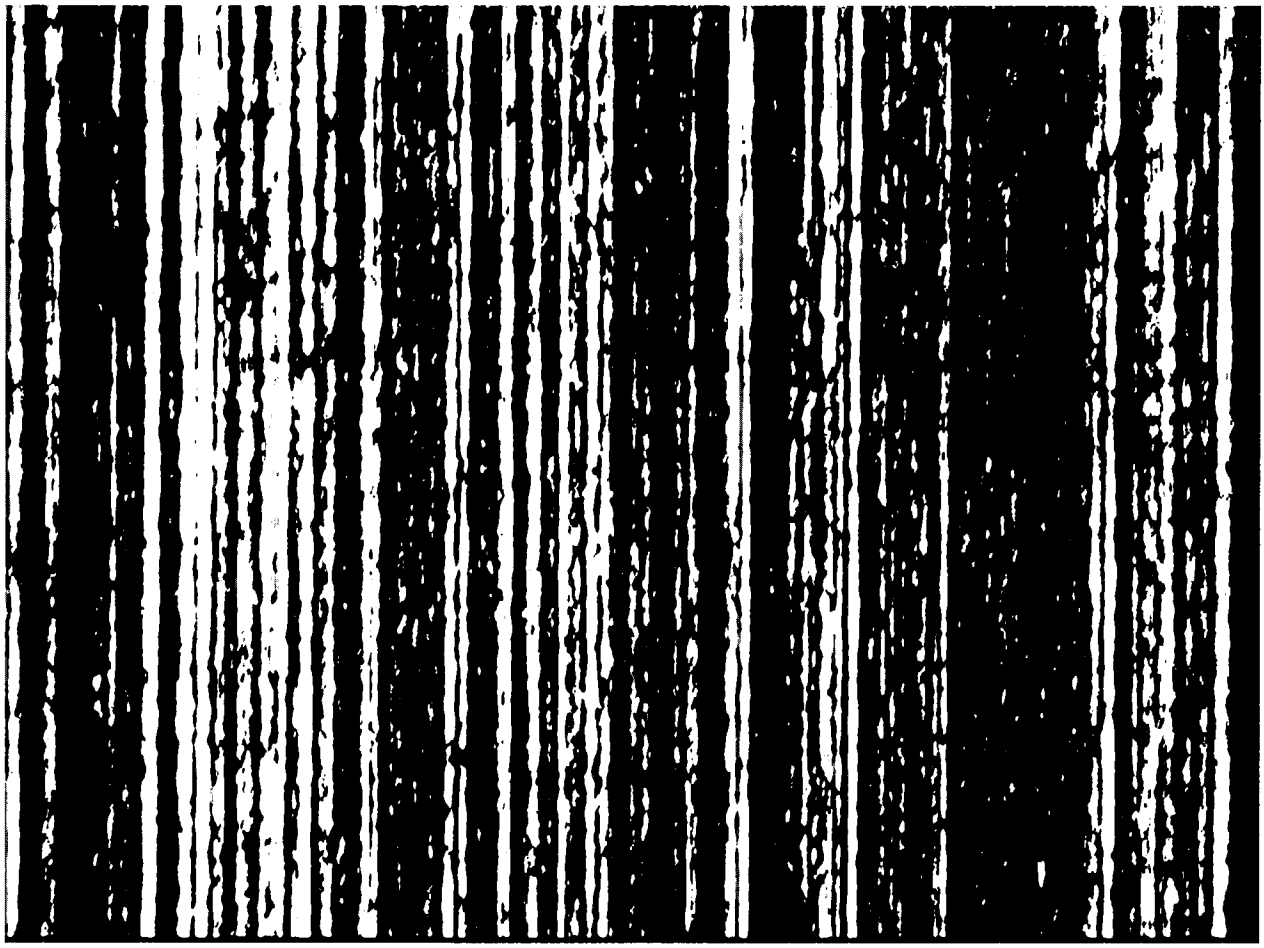

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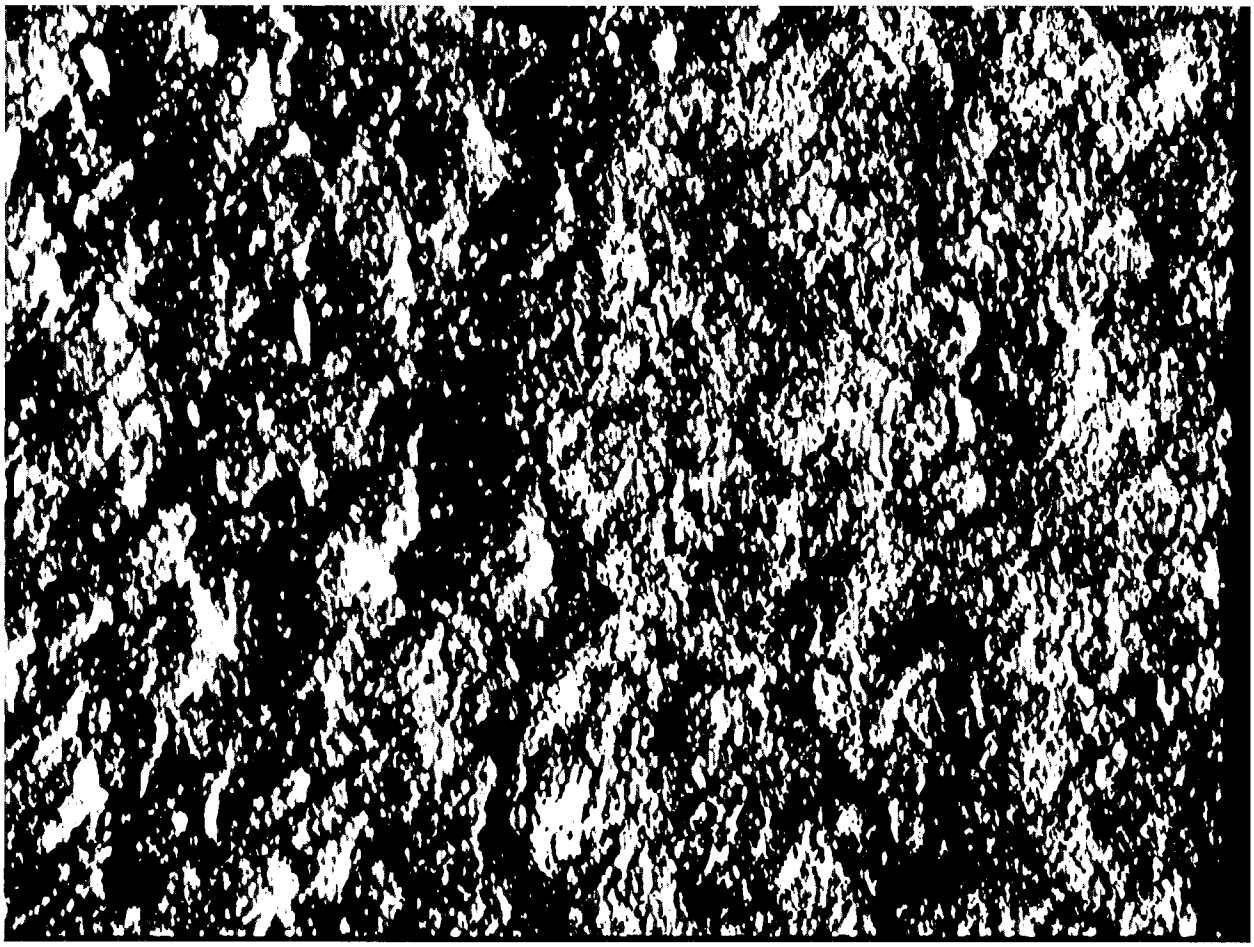

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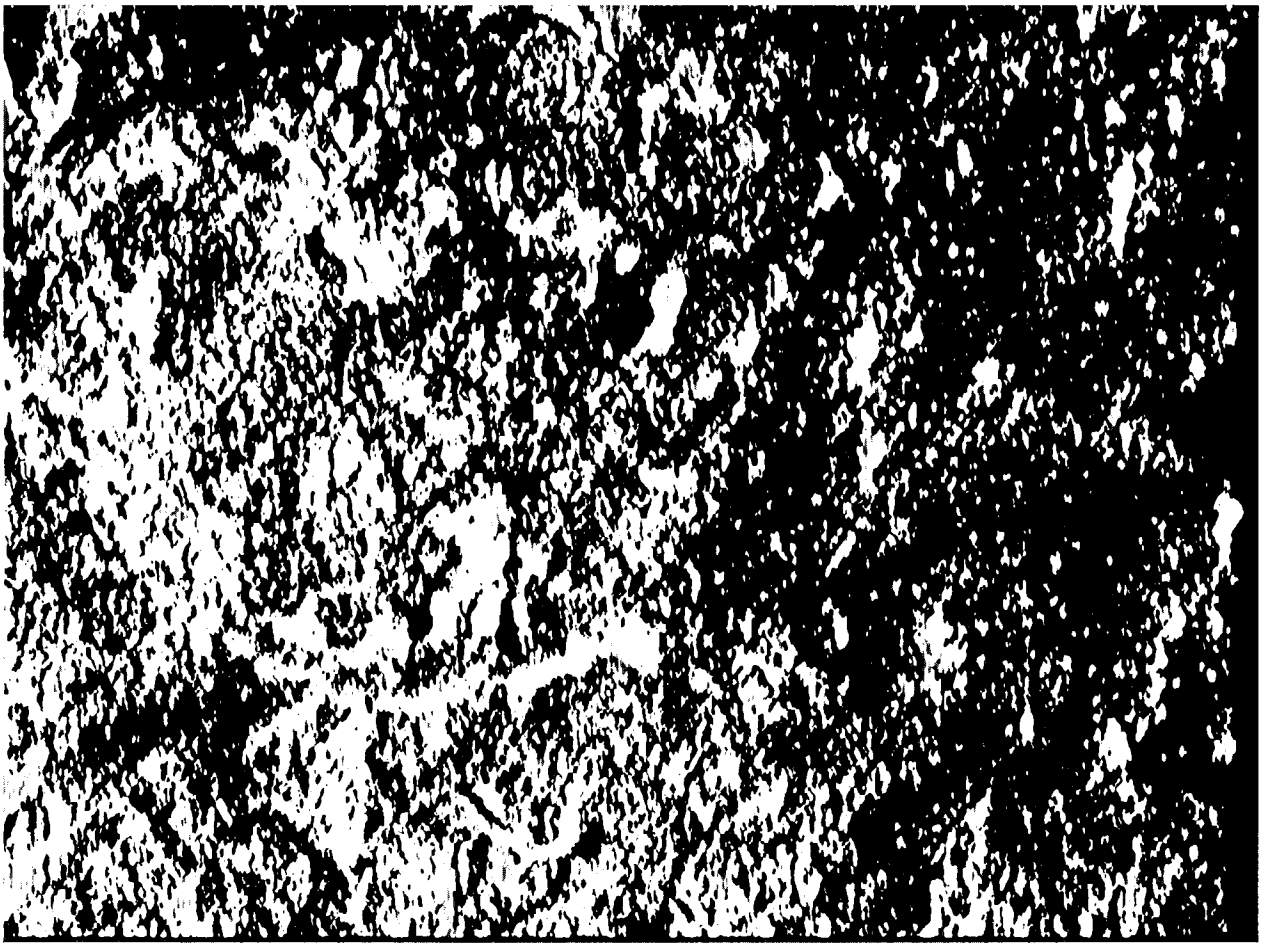

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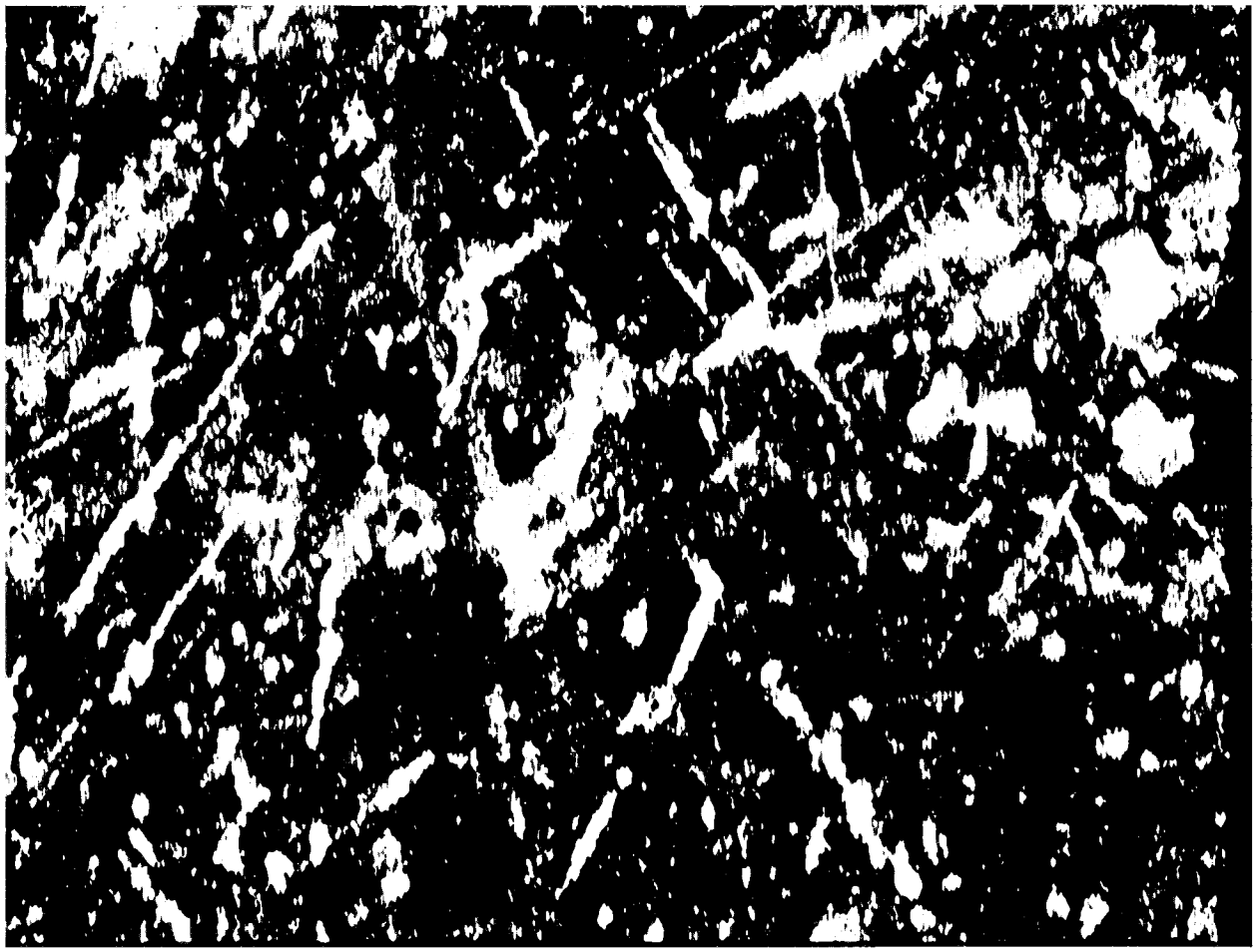

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